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c-Cbl Is Involved in Met Signaling in B Cells and Mediates Hepatocyte Growth Factor-Induced Receptor Ubiquitination

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Hepatocyte growth factor/scatter factor (HGF) and its receptor tyrosine kinase Met are key regulators of epithelial motility and morphogenesis. Recent studies indicate that the HGF/Met pathway also plays a role in B cell differentiation, whereas uncontrolled Met signaling may lead to B cell neoplasia. These observations prompted us to explore HGF/Met signaling in B cells. In this study, we demonstrate that HGF induces strong tyrosine phosphorylation of the proto-oncogene product c-Cbl in B cells and increases Cbl association with the Src family tyrosine kinases Fyn and Lyn, as well as with phosphatidylinositol-3 kinase and CrkL. In addition, we demonstrate that c-Cbl mediates HGF-induced ubiquitination of Met. This requires the juxtaplombud tyrosine Y1001 (Y2) of Met, but not the multifunctional docking site (Y14/15) or any additional C-terminal tyrosine residues (Y13–16). In contrast to wild-type c-Cbl, the transforming mutants v-Cbl and 70Z/3 Cbl, which lack the ubiquitin ligase RING finger domain, suppress Met ubiquitination. Our findings identify c-Cbl as a negative regulator of HGF/Met signaling in B cells, mediating ubiquitination and, consequently, proteasomal degradation of Met, and suggest a role for Cbl in Met-mediated tumorigenesis. The Journal of Immunology, 2002, 169: 3793–3800.

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2 Abbreviations used in this paper: HGF, hepatocyte growth factor; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; KD, kinase dead; NGF, nerve growth factor; PI-3K, phosphatidylinositol-3 kinase; WT, wild type.
Materials and Methods

Antibodies
mAbs used were anti-phosphotyrosine, PY20 (Affiniti, Nottingham, U.K.), and anti-hemagglutinin tag, 12CA5 (anti-HA) (C. de Vries, Department of Biochemistry, AMC, Amsterdam, The Netherlands). The rabbit polyclonal Abs used were: anti-ubiquitin (DAKO, Glostrup, Denmark); anti-human Met, C-12; anti-mouse Met, SP260; anti-Fyn, FYN3; anti-Lyn, 44; anti-CrkL, C-20; anti-Cbl, C-15 (all: Santa Cruz Biotechnology, Santa Cruz, CA); and anti-PI-3K p85 (Upstate Biotechnology, Lake Placid, NY).

Plasmids
The c-Cbl cDNA was a kind gift from W. Y. Langdon (University of Western Australia, Nedlands, Australia). pMT2-encoding HA-tagged human c-Cbl, v-Cbl, and 70Z/3 Cbl were generated from this cDNA by PCR. The constructs encoding Trk-Met (a chimeric receptor that consists of the extracellular domain of the nerve growth factor (NGF) receptor, Trk A, and the cytoplasmic domain of c-Met), either wild type (WT) or mutants of either tyrosine residue 1001 (Y12), 1232 and 1233 (kinase dead (KD)), 1347 (Y14), 1354 (Y15), 1347 and 1354 (Y14/15), or 1311, 1347, 1354, and 1363 (Y13–16), were a kind gift from W. Birchmeier (Max-Debrueck-Center for Molecular Medicine, Berlin, Germany) (39). pMT2-encoding HA-tagged ubiquitin was kindly provided by P. M. P. van Bergen en Negouwen (Department of Molecular Cell Biology, Utrecht University, Utrecht, The Netherlands).

Cell lines and transfectants
The Burkitt’s lymphoma cell line Namalwa-V3M has been described (34). The cells were cultured in RPMI 1640 in the presence of 10% fetal clone serum (HyClone Laboratories, Logan, UT) and 10% FCS (Integro, Zaan dam, The Netherlands). COS-7 cells were maintained in DMEM containing 10% FCS. Using DEAE-dextran, COS-7 cells were transiently transfected with 1 μg construct encoding Trk-Met, alone or together with 2 μg construct containing either HA-tagged c-Cbl, the oncogenic 70Z/3 Cbl and v-Cbl, or ubiquitin.

Immunoprecipitation and Western blot analysis
Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 10 μg/ml aprotinin (Sigma-Aldrich, St. Louis, MO), 10 μg/ml leupeptin (Sigma-Aldrich), 2 mM sodium orthovanadate, 5 mM EDTA, and 5 mM sodium fluoride. The lysates were cleared by centrifugation at 10,000 × g at 4°C for 20 min, followed by preclearance using protein A-Sepharose. The immunocomplexes were collected by adding the indicated Abs, precoupled to protein A-Sepharose, for at least 2 h. The immunoprecipitates were washed three times with lysis buffer, and the immunoprecipitated proteins were resolved by SDS-PAGE. The proteins were electrotransferred to nitrocellulose membranes. Detection of proteins by immunoblotting was performed using ECL lighting. For the immunodepletion experiments, the lysates were immunoprecipitated twice. The lysates remaining after the second immunodepletion and the immunoprecipitates obtained from the first immunoprecipitation were analyzed by Western blotting. Densitometric quantification analysis of Met was conducted on directly scanned images using National Institutes of Health Image 1.62 for Macintosh software. The protein levels of Met protein detected upon stimulation are expressed as a percentage of the amount of Met in unstimulated cells (100%). All values of Met have been adjusted for loading using extracellular signal-regulated kinase (ERK) as control.

Immune complex kinase assays
The Cbl immune complexes from unstimulated or HGF-stimulated cells were washed three times with lysis buffer, followed by washing twice with kinase buffer (50 mM HEPES (pH 7.5), 10 mM MgCl, 10 mM MnCl, and 1 μM sodium orthovanadate), suspended in 20 μl kinase buffer containing 10 μCi [γ-32P]ATP, and incubated for 30 min at room temperature. The proteins were separated on 10% SDS-PAGE, the gel was dried for 3 h, and the dried gel was autoradiographed at −80°C overnight.

Results

Cbl is strongly phosphorylated on tyrosine residues following HGF stimulation
We have recently demonstrated that activation of Met in Namalwa B cells leads to strong tyrosine phosphorylation of two proteins with molecular mass of 110–120 kDa (33, 34). The smaller of these proteins was shown to represent the Grb2-associated binder 1 (Gab1), an adaptor protein that can associate with the cytoplasmic docking site of Met (34). By performing immunodepletion experiments, we now identified the larger prominent phosphoprotein in the lysates of HGF-stimulated cells as c-Cbl (Fig. 1A). Immunoblotting of Cbl immunoprecipitates with Abs against phosphotyrosine confirmed that HGF stimulation leads to a rapid and transient phosphorylation of Cbl on tyrosine residues, peaking at 1 min and decreasing after 5 min (Fig. 1B).

HGF stimulation leads to enhanced association of c-Cbl with Fyn, Lyn, PI-3K, and CrkL
The above observations prompted us to explore the function of Cbl in Met signaling. Although Cbl itself lacks kinase activity, its characteristic modular structure enables it to act as a scaffold for various signaling molecules, including cytoplasmic tyrosine kinases (40). To determine whether HGF stimulation leads to changes in the kinase activity associated with Cbl, we conducted in vitro kinase assays. We observed that a low level of kinase activity was...
associated with Cbl immunoprecipitated from unstimulated B cells. However, HGF stimulation greatly increased the Cbl-associated kinase activity (Fig. 2). The 120-kDa in vitro phosphorylated protein present after stimulation with HGF represents c-Cbl itself, whereas the bands at 55–60 kDa may represent (auto) phosphorylated Src-family tyrosine kinases associated with c-Cbl. These kinases presumably are involved in the in vitro phosphorylation of c-Cbl and associated proteins.

To identify signaling molecules that dock on Cbl following HGF stimulation, the effect of HGF stimulation on the physical interaction with several candidate partners of Cbl was explored. These included the Src-family tyrosine kinases Fyn and Lyn, the p85 regulatory chain of PI-3K, and CrkL. We observed that these molecules all show a weak basal interaction with Cbl. However, upon stimulation with HGF, these interactions were either moderately (Fyn) or strongly (Lyn, CrkL, and PI-3K) enhanced (Fig. 3).

FIGURE 2. HGF stimulation induces an increase in the Cbl-associated kinase activity. Cbl immunoprecipitates collected from cells stimulated with HGF for the indicated time periods were phosphorylated in an in vitro kinase assay, as described in Materials and Methods. The arrow indicates the in vitro phosphorylated Cbl protein. The positions of prestained m.w. markers are indicated on the left side of the figure.

FIGURE 3. HGF stimulation leads to increased association of Cbl with Fyn, Lyn, CrkL, and the p85 subunit of PI-3K. Namalwa B cells were stimulated with HGF for the indicated time. The cells were lysed, and immunoprecipitates were collected using the indicated Abs. A, Increased association with Lyn and Fyn. Lyn and Fyn immunoprecipitates were subjected to immunoblotting using anti-Cbl Abs (upper panels). B, Increased association with CrkL and PI-3K. Cbl immunoprecipitates were subjected to immunoblotting using anti-CrkL and anti-p85 Abs (PI-3K) (upper panels). Equal loading of the samples was confirmed by restaining the blots with the same Abs used for immunoprecipitation (lower panels).

FIGURE 4. HGF stimulation induces ubiquitination and degradation of Met in B cells. A, HGF induces ubiquitination of Met. Namalwa B cells were stimulated with HGF for the indicated time. Anti-Met (C12) immunoprecipitates were immunoblotted with anti-ubiquitin Abs (upper panel) or, as a loading control, with anti-Met Abs (lower panel). B, HGF induces degradation of Met. Namalwa B cells were incubated in the presence or absence of HGF for the indicated time. Cell lysates were immunoblotted with anti-Met Abs (upper panel) or, as a loading control, with anti-ERK1 Abs (lower panel). C, The protein levels of Met in 4B were analyzed by densitometric quantification and, after correction using ERK as loading control, presented as the amount of Met protein relative to unstimulated cells (100%).
Hence, HGF stimulation does not only induce tyrosine phosphorylation of Cbl, but also enhances its ability to act as a docking protein for several important signaling molecules.

**Cbl plays a critical role in the Met ubiquitination**

The prominent phosphorylation of Cbl in response to HGF stimulation (Fig. 1), combined with the recent observation that Cbl acts as an E3 ubiquitin ligase for the epidermal growth factor (EGF) and platelet-derived growth factor receptors (41, 42), suggests that Cbl might be involved in the ubiquitination and degradation of Met. To address this hypothesis, we first assessed whether Met on B cells is ubiquitinated in response to HGF stimulation. Hence, Met immunoprecipitates from HGF-stimulated cells were analyzed for ubiquitination by immunoblotting. We observed that HGF stimulation leads to a rapid ubiquitination of c-Met, which was maximal at 5 min (Fig. 4A). Because the ubiquitination machinery adds multiple and variable numbers of ubiquitin moieties to a single target molecule, the polyubiquitinated Met species is detected as a smear rather than a distinct band (Fig. 4A). In addition to inducing ubiquitination, HGF stimulation also resulted in degradation of Met, which was clearly detectable from 5–10 min of incubation onward (Fig. 4, B and C). Hence, HGF stimulation of B cells leads to both ubiquitination and degradation of Met.

To explore the role of Cbl in Met ubiquitination, COS-7 cells were transfected with Trk-Met, a chimeric receptor that consists of the extracellular domain of the NGFR (Trk A) and the intracellular domain of Met, either alone or in combination with c-Cbl. After NGF stimulation, Trk-Met was immunoprecipitated, and its ubiquitination was analyzed. As shown in Fig. 5, cotransfection of c-Cbl clearly enhanced the ligand-induced ubiquitination of Trk-Met. By contrast, overexpression of the oncogenic Cbl variant v-Cbl, which only consists of the N-terminal 357 aa, did not enhance the ligand-induced ubiquitination of the transfected Trk-Met, but rather suppressed the (weak) ubiquitination mediated by endogenous Cbl (Fig. 5A). Moreover, similar results were obtained for the oncogenic mutant 70Z/3 Cbl, which only lacks a functional RING finger domain as a consequence of the deletion of aa 366–382 (Fig. 5B) (40). These findings demonstrate that c-Cbl is involved in Met ubiquitination, whereas the oncogenic v-Cbl and 70Z/3 Cbl are unable to mediate ubiquitination, but instead act in a dominantly negative fashion on endogenous c-Cbl.

**The juxtamembrane tyrosine residue 1001 (Y2), but not the multisubstrate docking site of Met, is required for receptor ubiquitination by Cbl**

Upon stimulation by HGF, the C terminus of Met is strongly phosphorylated on tyrosine residues. Autophosphorylation of tyrosine residues 1349 (Y14) and 1356 (Y15) of Met is critical for most biological responses (29, 43–47). These tyrosine residues serve as a multisubstrate docking site for several proteins, including Gab1, Grb2, PI-3K, phospholipase C, Src, Shc, SHP-2, and STAT-3. To assess whether this site is also involved in transducing signals leading to Met ubiquitination, we used Trk-Met mutated at Y14 and/or Y15. Whereas mutation of the kinase-regulatory tyrosines 1234 (Y8) and 1235 (Y9), which gives rise to a kinase dead Trk-Met (KD), resulted in a total abrogation of ligand-induced autophosphorylation and ubiquitination (Fig. 6A), NGF stimulation still resulted in a clear ubiquitination of the single (either Y14 or Y15) as well as double mutant (Y14/15) (Fig. 6B). This demonstrates that Y14 and Y15 are not required for Cbl-mediated ubiquitination of Met (Fig. 6B). This result was not due to functional redundancy by the presence of the tyrosines 1313 (Y13) and 1363...
As a Trk-Met mutant containing mutations in Y13–16, i.e., all four autophosphorylated residues of Met C-terminal of the kinase domain, was still readily ubiquitinated upon stimulation with ligand (Fig. 6C). Given this unexpected result, combined with the observed gain-of-function effect as a consequence of its mutation, i.e., the transition of epithelial cells to a fibroblastoid phenotype (39), we hypothesized that the juxtamembrane tyrosine residue Y1001 (Y2) might play an important role in Met ubiquitination. Interestingly, mutation of Y2 indeed resulted in the complete loss of ligand-induced Cbl-mediated ubiquitination of Met (Fig. 6D).
Discussion

We identified one of the most prominent phosphoproteins in lysates of HGF-stimulated B lymphoma cells as Cbl (Fig. 1), thus implicating Cbl in HGF/Met signaling. Recently, Cbl phosphorylation has also been observed upon HGF stimulation of the epithelial Madin-Darby canine kidney and Hela cells (48, 49) as well as in Tpr-Met-transformed fibroblasts (50). The multidomain docking protein p120 Cbl is the cellular homologue of the v-cbl oncogene from the murine Cas NS-1 retrovirus, which induces pre-B lymphomas and myeloid leukemias (51). Cbl is prominently tyrosine phosphorylated upon stimulation of a number of receptors, resulting in its interaction with Src homology 2 domain-containing proteins such as the p85 subunit of the PI-3K, the guanine nucleotide exchange factor Vav, and the Crk adaptor protein family (40). Indeed, HGF stimulation led to an increase in the amount of kinase activity associated with Cbl (Fig. 2), as well as an enhanced association between Cbl and Fyn, Lyn, the p85 chain of PI-3K, and CrkL (Fig. 3). Apart from binding to Cbl via their Src homology 2 domains, these proteins may also interact with Cbl via their Src homology 3 domains. This interaction with proline-rich regions on Cbl presumably is important for the stimulus-independent part of their Cbl association (Fig. 3) (52, 53).

Our observation that HGF stimulation leads to an enhanced association of Cbl with PI-3K as well as with CrkL, is of considerable interest. PI-3K is a central regulator of different biological processes induced by HGF, including adhesion and survival, and a specific PI-3K docking site has been located on Y1349 of Met (34). Our present findings suggest that association of PI-3K with Cbl (Fig. 3) might represent an alternative route for the regulation of PI-3K activity by HGF. CrkL is an adaptor protein with two Src homology 3 domains, which can specifically bind to the guanine exchange factor C3G, an activator of Rap-1 (54). Formation of a Cbl-Crk-C3G complex may provide a mechanism for coupling Met with the Rap-1 pathway, which has been implicated in integrin activation (55). Interestingly, we have recently shown that HGF induces activation of integrins in B cells (10). Cbl may play a critical role in this HGF-induced integrin activation, as suppression of Cbl expression by antisense Cbl resulted in a marked decrease in integrin activation (55–57).

Receptor ubiquitination and consequent degradation by the proteosomal/lysosomal pathway constitute an integral part of the regulation of receptor protein tyrosine kinase function (41, 58–60). Indeed, we observed that, following stimulation of B cells with HGF, Met is ubiquitinated and degraded (Fig. 4). This observation confirms and extends observations by Jeffers et al. (61), who reported HGF-induced degradation and polyubiquitination of Met in epithelial cells. Importantly, we now demonstrate that Cbl plays a key role in the negative regulation of Met signaling, by mediating receptor ubiquitination (Fig. 5). A number of studies have identified Cbl as an important negative regulator of protein tyrosine kinases. In Caenorhabditis elegans, the Cbl homologue SL-1 was shown to inhibit vulva development mediated by LET-23, a homologue of the mammalian EGF-R (62), whereas overexpression of Cbl in mammalian cells inhibits activation of the EGF and platelet-derived growth factor receptors and Janus kinase-STAT (63–65). Recently, in vitro studies revealed that the c-Cbl has intrinsic E3 ubiquitin-protein ligase activity (60). The RING finger domain of Cbl is critical for this regulatory function, as mutants of Cbl containing a complete (v-Cbl) or partial (70Z/3 Cbl) deletion, or a point mutation (Cys381-Ala) in the RING finger domain, are defective in promoting receptor tyrosine kinase ubiquitination. Indeed, also in our present study, the oncogenic mutants v-Cbl and 70Z/3 Cbl failed to induce ubiquitination of Met, but rather had a dominant-negative effect on the ubiquitination induced by endogenous Cbl (Fig. 5). This is further supported by the recent finding that expression of 70Z/3 Cbl in Madin-Darby canine kidney cells results in an epithelial-mesenchymal transition, which resembles the effect of HGF stimulation (49). Thus, expression of these oncogenic mutants of Cbl might result in overexpression and constitutive activation of Met, leading to Met-mediated tumorigenesis.

The tyrosine residues Y14 and Y15 play a critical role in virtually all Met-mediated biological responses. These residues serve as docking sites for multiple signalling molecules, including Gab1, Grb2, PI-3K, phospholipase C, Src, Shc, SHP-2, and STAT-3 (29, 43–47). Interestingly, we observed that this multisubstrate docking site of Met is not required for ubiquitination by Cbl. Mutation of neither the tyrosines Y14 and/or Y15, nor of all autophosphorylated residues in the C-terminal domain of Met, i.e., Y13–16, interfered with NGF-induced ubiquitination of Trk-Met (Fig. 6, B and C). By contrast, Met ubiquitination was dependent on the integrity of the juxtamembrane tyrosine residue Y2 (Fig. 6D). These data support a recent study that demonstrated a role for Cbl and Y2 in ligand-independent ubiquitination of Met (66). In addition, in this study we have shown that Cbl and Y2 are also critical in Met ubiquitination induced by ligand (Figs. 5 and 6D), that the oncogenic mutants 70Z/3 Cbl and v-Cbl act in a dominant-negative fashion (Fig. 5), and that ubiquitination of Met does not depend on its C-terminal tyrosine residues (Y13–16), which include the docking site of Met (Y14/15) (Fig. 6, B and C). Previously, it has been reported that mutation of residue Y2 of Met leads to a gain-of-function resulting in constitutive scattering and fibroblastoid morphology of epithelial cells (39). Our data suggest that this may be due to a defect in Cbl-mediated Met ubiquitination. In addition, although most germline and sporadic Met mutations in human tumors involve the kinase domain and result in enhanced kinase activity upon stimulation with ligand (20–22), recently mutations have also been reported in the juxtamembrane portion of Met (24). Met carrying such a missense mutation at P1099S (P989 in mouse) was not constitutively active, but showed increased and persistent Met phosphorylation after HGF treatment. This activating mutation is localized in a PEST (amino acid residues Pro, Glu, and/or Asp, Ser, and Thr)-like sequence, which has been implicated in ubiquitination (24). Hence, tyrosine Y2 and adjacent sequences in the juxtamembrane domain of Met appear to play a critical role in the negative regulation of Met by Cbl. Taken together, these findings identify Cbl as negative regulator of Met and suggest that defects in this negative regulation, caused by mutations in either Cbl or Met, may contribute to tumorigenesis.

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