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Specific Grb2-mediated Interactions Regulate Clathrin-dependent Endocytosis of the cMet-tyrosine Kinase*[S]

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Lysosomal degradation of the receptor-tyrosine kinase cMet requires receptor ubiquitination by the E3 ubiquitin ligase Cbl followed by clathrin-dependent internalization. A role for Cbl as an adaptor for cMet internalization has been previously reported. However, the requirement for Cbl ubiquitin ligase activity in this process and its mode of recruitment to cMet has yet to be determined. Cbl can directly bind cMet at phosphotyrosine 1003 or indirectly via Grb2 to phosphotyrosine 1356 in the multisubstrate binding domain of cMet. The direct binding of Cbl with cMet is critical for receptor degradation and not receptor internalization. Here we show a strict requirement for Grb2 and the ubiquitin ligase activity of Cbl for cMet endocytosis. Receptor internalization was impaired by small interfering RNA depletion of Grb2, overexpression of dominant negative Grb2 mutants, and point mutations in the cMet multisubstrate docking site that inhibits the direct association of Grb2 with cMet. The requirement for Grb2 was specific and did not involve the multiadaptor Gab1. cMet internalization was impaired in cells expressing an ubiquitin ligase-deficient Cbl mutant or conjugation-deficient ubiquitin but was unaffected in cells expressing a Cbl mutant that is unable to bind cMet directly. Expression of a Cbl-Grb2 chimera rescued impaired cMet endocytosis in cells depleted of endogenous Grb2. These results indicate that the ubiquitin ligase activity of Cbl is critical for clathrin-dependent cMet internalization and suggest a role for Grb2 as an intermediary linking Cbl ubiquitin ligase activity to this process.

Activation of the receptor-tyrosine kinase (RTK)[2] cMet by binding to its physiological ligand hepatocyte growth factor (HGF) results in increased cell proliferation and cell motility, processes critical for embryonic development, tissue regeneration, and homoeostasis (for review, see Ref. 1). Persistent cMet signaling occurs in many human cancers, correlating closely with early stage invasion, dissemination, and metastases (for review, see Refs. 1 and 2). Once activated by ligand binding, cMet is normally endocytosed exclusively through clathrin-coated pits (3, 4) and then inactivated by lysosomal degradation (3, 5). Although several mechanisms likely contribute to sustained cMet signaling in cancer (6–12), dysregulated receptor internalization and degradation have recently emerged as novel mechanisms for cMet-induced cell transformation and the increased neoplastic growth of human tumors (13–16).

Endocytic studies on other RTKs, most notably the epidermal growth factor receptor (EGFR), suggest that modification of the endocytic trafficking machinery by the activated receptor and/or its associated signaling molecules regulates receptor internalization and degradation. For example, the signaling adaptor Grb2 has been shown to function as an important initiator of EGFR endocytosis (17–20). Small interfering RNA (siRNA)-mediated depletion of Grb2 and overexpression of Grb2-SH3 mutants deficient in binding downstream signaling molecules blocks the recruitment of EGFR into clathrin-coated pits and subsequent receptor internalization, with negligible effects on the downstream activation of mitogen-activated protein kinase or phosphatidylinositol 3-kinase (19). Grb2 functions to regulate EGFR internalization through the recruitment of the E3 ubiquitin ligase Cbl (20–22). However, it remains unclear if the requirement for Grb2 on EGFR endocytosis is mediated solely through interactions with Cbl, functions at the level of EGFR ubiquitination, involves additional Grb2-dependent interactions, or extends to other RTKs.

cMet and the EGFR are prototypic members for distinct RTK subfamilies and as such exhibit unique structural and functional characteristics. In contrast to the EGFR, cMet is a heterodimer composed of an extracellular α chain that is disulfide-linked to a transmembrane β chain that contains cytoplasmic tyrosine kinase activity (23–25). The extracellular domain of cMet contains a distinctive β-propeller fold that mediates ligand binding (26). Ligand binding induces the autophosphorylation of cMet and EGFR at multiple tyrosines in their respective C-terminal regions enabling the recruitment and subsequent activation of shared downstream adaptor and signaling molecules. The EGFR possesses six tyrosine transphosphorylation sites dispersed throughout the C-terminal region (for

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[2] The abbreviations used are: RTK, receptor-tyrosine kinase; HGF, hepatocyte growth factor; cMet, HGF receptor; InlB, internalin B; Alexa-InlB, Alexa-labeled InlB; EGFR, epidermal growth factor (EGF) receptor; Tfn, transferrin; siRNA, small interfering RNA; wt, wild type; IP, immunoprecipitation; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; FACS, fluorescence-activated cell sorter; MFI, mean fluorescence intensity; EYFP, enhanced yellow fluorescent (YFP) protein; KinD-cMet, kinase-dead cMet; UbR, mutant ubiquitin.

review, see Ref. 27), whereas cMet contains two key tyrosine phosphorylation sites at positions 1349 and 1356 in a unique multisubstrate-docking site, that function to concomitantly activate multiple downstream signaling pathways (28, 29). In contrast to cMet, which contains one binding site for the signaling adaptor Grb2 at phosphotyrosine 1356 (28, 30), Grb2 binds EGFR directly at two sites (31). Moreover, Grb2 appears to play a minor role in signaling events downstream of ligand activated cMet *in vivo* (32–34) when compared with the EGFR (34). The adaptor protein Gab1 binds to two sites in the multisubstrate-docking site of cMet; directly at phosphotyrosine 1349 or indirectly via the adaptor Grb2 to phosphotyrosine 1356 (35–38). Gab1 recruitment is essential for the induction of cell motility, branching morphogenesis, and the formation of crypts in three-dimensional matrices, hallmarks of cMet signaling (29, 33, 39–41). Given these differences in adaptor recruitment and biological activity, some differences may exist in the regulatory mechanisms controlling cMet *versus* EGFR internalization.

The E3 ubiquitin ligase Cbl has been shown to mediate the ubiquitination of ligand-activated cMet (4, 5, 14, 42, 43), receptor internalization (4), and degradation (13, 14). Cbl recruitment via its tyrosine kinase binding domain to phosphotyrosine 1003 of cMet has been shown to contribute to receptor ubiquitination (13, 14, 42) and degradation in lysosomes (13, 14, 43). Cbl has been shown to function as an adaptor important for cMet internalization through clathrin-coated pits via the recruitment of the endophilin-CIN85 complex (4). However, a requirement for Cbl ubiquitin ligase activity and its mode of recruitment for cMet internalization was not examined. In this study we report that the ubiquitin ligase activity of Cbl is important for clathrin-mediated cMet internalization. Moreover, Grb2 bound directly to the cMet multisubstrate docking site acts as an intermediary for Cbl ubiquitin ligase activity in these events.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and Plasmids—All general reagents were obtained from Fisher or Sigma-Aldrich unless indicated otherwise. Recombinant human HGF was purchased from PeproTech Inc, Rocky Hill, NJ. EZ-link sulfo-NHS-SS-biotin, D-salt dextran, and spin columns were purchased from Pierce. Texas Red-labeled-transferrin (Tfn) and antibody labeling kits were obtained from Invitrogen. The following antibodies were purchased as indicated; Shc (BD Biosciences), anti-human HGF (R&D Systems Inc, Minneapolis, MN), transferrin receptor (Invitrogen), anti-β-actin and anti-phosphotyrosine clone PY-20 (Sigma-Aldrich), ubiquitin (P4D1), Cbl (C-15), Grb2 (C-23), cMet C-12, and cMet C-28 (Santa Cruz Biotechnologies), anti-Gab1 CT, phospho-Met Tyr-1234, Tyr-1235 (UpState Biotechnology), Gab1, phospho-Gab1, c-Jun N-terminal kinase (JNK), phospho-JNK, p42/p44 MAPK, phospho p42/p44, MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase), and phospho-MEK (Cell Signaling Technology), the monoclonal GFP antibody (JL-8) (BD Biosciences Clontech), peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.), and Alexa^{488} or Alexa^{594}-labeled goat anti-mouse, anti-rabbit, or anti-rat secondary antibodies (Invitrogen). A pLXSN plasmid encoding wild type (wt)-cMet was previously reported (44). pLXSN plasmids encoding the full-length Met mutants N1358H and Y1349F,Y1356F were generously provided by Pascal Peschard and Morag Park (McGill University). The K1110A allele encodes a Met receptor lacking kinase activity (45, 46). The K1110A mutation was generated through a PCR-based SOEing approach (47) using wild type cMet cDNA as a template, *Pfu* DNA polymerase, and the primers 5′-CAAGCTAGCCACAGCACAGTG-3′, 5′-CCCATCCTAACTAGTGGGGAC-3′, 5′-CACTGTGCTGTGGCTAGCTTG-3′, and 5′-GCTCTAGAACTAGTGGATCCC-3′. The final ~1.3-kilobase PCR product was subcloned between the SpeI and XcmI sites of pLXSN-Met-wt, resulting in a full-length Met gene with the K1110A mutation and verified by DNA sequencing. The various Met alleles were subcloned from the plasmid pLXSN into the murine stem cell virus vector pMSCVpuro (BD Biosciences) for retroviral-mediated transfection in the target cell lines. pYFP-N1 plasmids encoding Grb2 and Grb2-mSH3 were kindly provided by Lawrence Samelson (National Institutes of Health) (48). The pEGFP plasmids encoding Cbl, Cbl-SH2, Cbl-SH2/R86A, Grb2-SH2, and Grb2/R86A were kindly provided by Alexander Sorkin (University of Colorado Health Sciences Center) (17, 22). pcDNA3-Myc-UbR and pcDNA3-Myc-UbRL8A/I44A were kind gifts from Inger Madshus (Norwegian Radium Hospital) (18). Plasmids encoding hemagglutinin-tagged Cbl (wt), 70Z-Cbl, and Cbl-G306E were kindly provided by Jannie Borst (The Netherlands Cancer Institute) (20).

Cell Lines—The H10 cell line derived from kidney epithelial cells from embryos of cMet null −/− mice (49) was a generous gift of Dr. Lloyd Cantley (Yale University School of Medicine). Polyclonal H10 cell lines expressing cMet alleles were generated by retroviral transfection essentially as described (50), except that 1–5 μg/ml puromycin was used for selection, and limiting dilution was not performed. Cell lines were analyzed for cMet expression by Western blotting with the anti-cMet antibody DL-21 (Upstate Biotechnology), and those cell lines that exhibited similar cMet levels were chosen for further analysis. The parental H10 cMet null −/− cell line was maintained in Dulbecco's modified Eagle's medium/F-12(1:1) with 10% fetal bovine serum. H10 derivatives stably expressing wild type or mutant cMet alleles were grown in the same medium containing 5 μg/ml puromycin. Human mammary epithelial cells (T47D) stably expressing full-length human cMet (T47D/cMet) were a generous gift of Morag Park (McGill University) and were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with 400 μg/ml G418.

siRNAs and Cell Transfections—For siRNAs depletion studies, cells were grown on coverslips in 60-mm plates and transfected with Smartpools™ containing four siRNA duplexes for Gab1, Grb2, Shc, or a control siRNA using Lipofectamine 2000 reagent (Invitrogen). Typically each coverslip of cells was transfected with 200 pmol of siRNA and 8 μl of Lipofectamine 2000. T47D/cMet cells were routinely cultured on coverslips coated with 100 μg/ml polylysine before experimentation. All experiments were routinely performed 72 h after siRNA transfection.

Cell transfections using plasmids were performed as previously described (3).

Immunoprecipitation and GST Pulldown Assays—For immunoprecipitation (IP) studies cells were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and lysed on ice in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) with protease inhibitors (2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin) and phosphatase inhibitors (10 mM NaF, 2 mM Na_3VO_4). Cell lysates were cleared by centrifugation at 12,000 $\times$ *g* at 4 °C, and then 500–1000 μg of each lysate was incubated with 5 μg of primary antibody overnight at 4 °C. Antibody-protein complexes were precipitated with 50 μl of protein A/G-agarose solution (Pierce) by rotation at 4 °C for 4 h. The protein-beads complex were collected by centrifugation at 1000 $\times$ *g* for 5 min, washed with lysis buffer 3 times, and then resuspended in SDS loading buffer and fractionated by SDS/PAGE. For GST pulldown assays, the cell lysates were prepared in lysis buffer (1% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 10 mM NaF, 2 mM Na_3VO_4). Lysates (0.5–1.0 mg of protein) were incubated with the appropriate GST fusion proteins prebound to glutathione-Sepharose beads (Sigma) overnight at 4 °C. Beads were washed three times in lysis buffer followed by a final wash in 10 mM Tris-HCl, pH 7.4, then analyzed by SDS/PAGE.

Cell Surface Biotinylation and Western Analysis—The biotinylation and receptor internalization assays were described previously (3). Briefly, cells were surface-biotinylated at 4 °C for 30 min with EZ-link NHS-SS-biotin (Pierce). Surface-expressed proteins were isolated directly using streptavidin-agarose beads as described by the manufacturer (Pierce) and identified by Western analysis. Western analysis was performed using ECL (GE Healthcare), and the resulting digitized blots were quantified using AlphaEase Version 3.1.2 Software (Alpha Innotech Corp.).

Flow Cytometry Analysis—cMet internalization from the plasma membrane was measured by assaying the residual amount of immunoreactive receptor accessible at the cell surface to a monoclonal antibody recognizing an extracellular epitope. Briefly, cells grown to 80% confluence on 60-mm dishes were sera-starved for 4–6 h and then stimulated with 100 ng/ml internalin B (InlB) at 37 °C under steady state conditions to drive cMet internalization. At the indicated times, receptor endocytosis was terminated by placing the cells on ice. The cells were immediately rinsed with ice-cold PBS, and residual surface-bound InlB was stripped using three consecutive 5-min ice-cold acid washes (Dulbecco's modified Eagle's medium, pH 3.5). Cells were detached with PBS containing 5 mM EDTA and then resuspended in ice-cold FACS buffer (PBS containing 2% fetal bovine serum). Cells were incubated with anti-cMet antibody (AF276, R&D System, MN) in FACS buffer at 4 °C for 1 h, washed 3 times with ice-cold FACS buffer, and then incubated with $Alexa^{488}$-conjugated secondary antibody in FACS buffer at 4 °C for 30 min. After 3 ice-cold washes with FACS buffer, the cells were analyzed using a FACS Canto flow cytometer or fixed with 2% paraformaldehyde, PBS for later analysis. Cell viability, as determined by the exclusion of propidium iodide, routinely exceeded 95%. 20,000 cells were analyzed for each sample in triplicate for each condition in each experiment. The mean fluorescence intensity (MFI) of the cells at each time point was analyzed and compared with a negative control using control antibody only. The MFI values for each condition were averaged, and the S.E. was calculated across all experiments. The relative percentage of residual cell surface cMet at each time point (t_x) was calculated relative to the MFI of cells without internalization (t_0) as (MFI t_x − MFI control antibody only/MFI t_0 − MFI control antibody only) $\times$ 100.

InlB Labeling, Confocal Microscopy, and Analysis—The purification and labeling of recombinant His_6-tagged InlB has been described in detail elsewhere (3). For confocal microscopy cells grown on coverslips were incubated in media containing 5.0 μg/ml $Alexa^{594}$-labeled Tfn with 100 ng/ml InlB or HGF for 10 min at 37 °C, then fixed immediately after each experiment using 4% paraformaldehyde (Ted Pella Inc.) in PBS. Residual paraformaldehyde was quenched using 50 mM NH_4Cl/PBS, and cells were then permeabilized with 0.05% (w/v) saponin (or 0.1% Triton X-100, PBS for HGF staining) for 20 min and then blocked with 10% goat serum in PBS. All antibody dilutions were performed in 5% goat serum, PBS with 0.05% saponin for 1 h. In studies staining for HGF, saponin was omitted from the buffers. Coverslips were mounted and observed using a Zeiss LSM 510 confocal microscope with a 63$\times$ oil (1.4 NA) immersion objective. Samples were visualized with the 488- and 543-nm laser lines and emission filter sets at 505–530 nm for YFP and $Alexa^{488}$ detection or 585–615 nm for Texas Red and $Alexa^{594}$ detection, respectively. Figure presentation was accomplished in Adobe Photoshop Version 6.0. Quantification of co localization and internal fluorescence intensity were accomplished using Metamorph v5.0 (Molecular Devices) as described previously (3). All pixel intensity levels were normalized relative to control values, expressed as a percentage ± S.E., and differences were statistically verified by analysis of variance using GraphPad Prism Software (GraphPad Software Inc.).

RESULTS

The Tyrosine Kinase Activity and the Docking Site Phosphotyrosines 1349 and 1356 of cMet Are Required for Ligand-induced Internalization—All of the known signaling cascades downstream of activated cMet are mediated by autophosphorylation of two key tyrosines located at positions 1349 and 1356 in the novel multisubstrate docking site (28). To determine whether autophosphorylation of these tyrosine residues is critical for cMet internalization, wt or mutant cMet was expressed in cells immortalized from the kidneys of cMet null (−/−) mice. These epithelial $cMet^{-/-}$ cells undergo chemotaxis and branching morphogenesis in response to EGF and transforming growth factor-α but not HGF, indicating that cMet signaling is specifically abrogated in these cells (49). We generated several cell lines stably expressing moderate levels of wt or mutant cMet, including tyrosine kinase-deficient K1110A cMet (KinD-cMet) and the multisubstrate-docking site mutant Y1349F,Y1356F (YF-cMet), which is deficient in Gab1 and Grb2 binding. siRNA-mediated depletion of clathrin heavy chain inhibited wt cMet internalization (supplemental Fig. S1),

confirming that cMet internalization is clathrin-dependent in these cells, consistent with our previous studies using T47D/cMet and Vero cells (3).

We previously reported that the soluble form of the InlB protein of *Listeria monocytogenes* mimics HGF-induced cMet internalization and degradation (3). In addition to being presented as a soluble protein, InlB is also found anchored to the cell surface of bacteria (51). The surface-anchored form of InlB mediates bacterial uptake into host cells (5), whereas soluble InlB is thought to modulate host transcriptional responses (52, 53). We previously reported that HGF and soluble InlB are internalized with cMet via a clathrin-dependent mechanism (3). Interestingly, evidence indicates that the internalization of soluble InlB or HGF probably occurs through a mechanism that differs from that utilized by bacteria expressing surface-anchored InlB. Although the uptake of *Listeria* and cMet activated by soluble ligands involves select components of clathrin-coated pits (5), *Listeria* entry requires phosphatidylinositol 3-kinase activity (54), whereas the internalization of soluble HGF or InlB does not depend on this kinase (3). Because soluble InlB and HGF are internalized through identical pathways, we used InlB as an initial ligand to examine the molecular control of cMet endocytosis and then validated key findings using the physiological ligand HGF.

Western analysis of cell lysates prepared from the stable cell lines expressing wt or mutant cMet confirmed that treatment with InlB for 15 min at 37 °C caused phosphorylation of wt and the YF-cMet mutant on tyrosine residues 1234 and 1235 in the activation loop but not on kinase-dead cMet (KinD-cMet) (Fig. 1*A*). Ubiquitination of wt cMet but not KinD-cMet was increased in response to InlB. A low level of ubiqitinated YF-cMet was detected under these conditions, consistent with the direct association of Cbl to phosphotyrosine 1003 of cMet, independently of Grb2 binding (14). Cbl phosphorylation was not detected in unstimulated cells or in cells stably expressing KinD-cMet after treatment with ligand (Fig. 1*A*). In keeping with previous results (14, 36), cMet-mediated phosphorylation of Cbl was reduced in cells expressing YF-cMet relative to cells expressing wt cMet. This is consistent with a role for Grb2 in recruiting Cbl to cMet via phosphotyrosine 1356. Surface biotinylation assays were used to confirm the cell surface expression of wt and mutant cMet in each of the polyclonal cell lines (Fig. 1*B*). Cells were surface-biotinylated at 4 °C for 30 min using NHS-SS-biotin. Cell lysates were then prepared, and biotinylated proteins were isolated by streptavidin pulldown and analyzed by Western blots. Comparable levels of wt and mutant cMet were detected at the cell surface of the cell lines (Fig. 1*B*). No cMet was detected in the parental null cells, consistent with previous reports (49).

Using a GST pulldown assay, we verified that Grb2 bound to wt cMet in response to InlB but not to YF-cMet or KinD-cMet (Fig. 1*C*). No Grb2 binding was detected in the absence of ligand, confirming that Grb2 binding to wt cMet was specific and dependent on cMet autophosphorylation. Similarly, Gab1 phosphorylation in response to InlB was detected in null cells expressing wt cMet but not in cells expressing the YF- or KinD-cMet mutants (Fig. 1*D*), consistent with previous reports using fibroblasts and HEK293 cells (35, 55). Together, these data confirm that the recruitment of Gab1 and Grb2 to cMet is disrupted in the YF- and KinD-cMet mutants but not in cells expressing wt cMet.

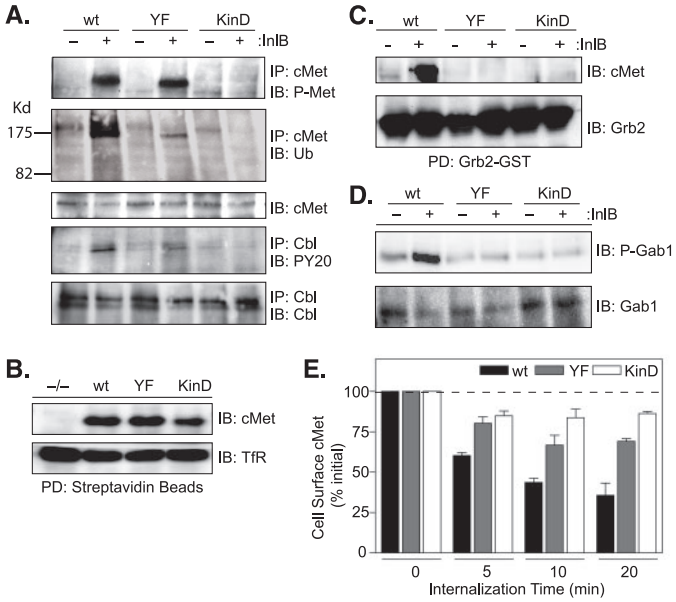

FIGURE 1. **cMet internalization requires phosphotyrosines 1349 and 1356.** *A*, cMet signaling in stable cMet null (−/−) cells expressing wt, KinD, or a mutant deficient in Grb2 and Gab1 binding (*YF*) was examined after incubation with (+) or without (−) 100 ng/ml InlB for 15 min at 37 °C by IP and Western analysis (*IB*) for expression of total protein (cMet, Cbl), cMet ubiquitination (*Ub*) and phosphorylation (*P-Met*), and Cbl phosphorylation using the anti-phosphotyrosine antibody PY20. The positions of molecular mass markers are indicated (kDa). *B*, pulldown (*PD*) surface biotinylation assays verified that wt and mutant cMet were expressed at comparable levels on the cell surface. *C*, Grb2 binding was detected in null (−/−) cells stably expressing wt but not mutant cMet in response to treatment with 100 ng/ml InlB. *D*, Gab1 was phosphorylated in response to treatment with (+) 100 ng/ml InlB in cells expressing wt but not mutant cMet. *E*, cMet null (−/−) cells expressing wt or the indicated cMet mutants were left untreated (0 min) or treated with 100 ng/ml InlB for 5, 10, or 20 min at 37 °C. The cells were chilled to 4 °C, and cMet on the cell surface was specifically labeled with anti-cMet antibody and quantified by flow cytometry. Results represented the mean fluorescent intensities normalized to untreated cells under each experimental condition from triplicate experiments. *Bars* represent the means for data across all experiments with S.E.

We next examined ligand-induced internalization of wt and mutant cMet using a flow cytometric method that measures the amount of immunoreactive receptor remaining on the cell surface after ligand treatment. Briefly, cMet null cells stably expressing wt or mutant cMet were incubated in the presence or absence of InlB (100 ng/ml) for increasing periods of time at 37 °C. After incubation, the cells were then chilled on ice to prevent further receptor trafficking, and cMet remaining on the cell surface was labeled specifically with a cMet-specific antibody under non-permeabilized conditions. Flow cytometry analysis was then used to quantify the relative number of immunoreactive receptors remaining on the cell surface under these conditions (Fig. 1*E*). All cell populations expressed similar levels of surface cMet under control conditions (*i.e.* no receptor internalization at 4 °C), consistent with their initial selection based on similar levels of cell surface receptor expression (data not shown). As expected, reduced levels of wt cMet were detected on the cell surface relative to cells incubated in the absence of InlB, consistent with ligand induced-receptor internalization. In contrast to wt cMet, higher cell surface levels of

YF-cMet and KinD-cMet were routinely detected at each time point. After an initial decrease in detected cell surface levels of KinD-cMet and YF-cMet at 5 and 10 min, respectively, receptor levels remained constant thereafter for up to 20 min of ligand treatment. The initial reduction in the surface levels of cMet could be the result of the constitutive internalization of cMet consistent with our earlier studies (3) or steric hindrance of antibody binding to cMet. We confirmed our flow cytometry studies using Alexa-labeled InlB (Alexa-InlB) as a ligand to examine the internalization of wt and mutant cMet by confocal microscopy (supplemental Fig. S2). After incubation for 10 min at 37 °C in media containing Alexa-InlB, internalized ligand was detected with co-internalized Texas Red-labeled Tfn in cells expressing wt cMet. Conversely, decreased levels of internalized InlB were detected in cells expressing KinD cMet or the cMet mutant lacking a functional Gab1/Grb2 binding site (YF). Rather, large spots of InlB fluorescence were detected on the surface of cells expressing YF-cMet. Clathrin-mediated Tfn uptake was unaffected in null cells expressing YF-cMet or KinD-cMet, consistent with a specific block in cMet internalization rather than saturation of the clathrin-dependent endocytic machinery (supplemental Fig. S1). Thus, activation of the cMet-tyrosine kinase activity and subsequent recruitment of one or more of the proteins that are known to associate with phosphotyrosines 1349 and 1356 are required for cMet internalization through clathrin-coated pits.

Grb2 Is Required for cMet Internalization—Our data indicating the dependence on phosphotyrosines 1349 and 1356 in the multisubstrate docking region for cMet internalization raised the possibility that the adaptors Grb2 and/or Gab1 could be required for this process. To test this idea we used specific siRNAs to deplete T47D/cMet cells of endogenous Grb2. T47D/cMet cells are a human mammary epithelial cell line that stably expresses human cMet on their surface and have been extensively used on studies examining cMet signaling and endocytosis in the past (3, 13, 14, 44). Endogenous Grb2 was weakly detected by Western analysis in T47D/cMet cells transfected with Grb2-siRNAs (Fig. 2*A*). Conversely, comparable levels of endogenous transferrin receptor, actin, and cMet were observed in Grb2-depleted T47D/cMet cells as well as untransfected cells, and T47D/cMet cells transfected with a control siRNA, demonstrating the specificity of the siRNA treatment. Western analysis confirmed that siRNA-mediated depletion of Grb2 did not reduce cMet phosphorylation in response to InlB (10 min at 37 °C) (Fig. 2*B*). Conversely, cMet ubiquitination and Cbl phosphorylation were reduced by Grb2 depletion. siRNA-treated cells were coincubated with Tfn and HGF or Alexa-InlB for 10 min at 37 °C, and ligand internalization was measured by confocal microscopy. cMet internalization in response to HGF or InlB was reduced 51.3 ± 4.85 and 74.4 ± 2.87%, respectively, in Grb2-depleted cells *versus* cells transfected with a control siRNA (Fig. 2*C*). Comparable levels of co-internalized Tfn were detected in control and Grb2-depleted T47D/cMet cells, indicating that loss of Grb2 led to a specific block in cMet internalization and not a general defect in clathrin-mediated endocytosis (Fig. 2*C* and supplemental Fig. S3). siRNA-mediated Grb2 depletion caused a comparable block in cMet endocytosis in cMet null cells stably expressing wild type receptor, indicating

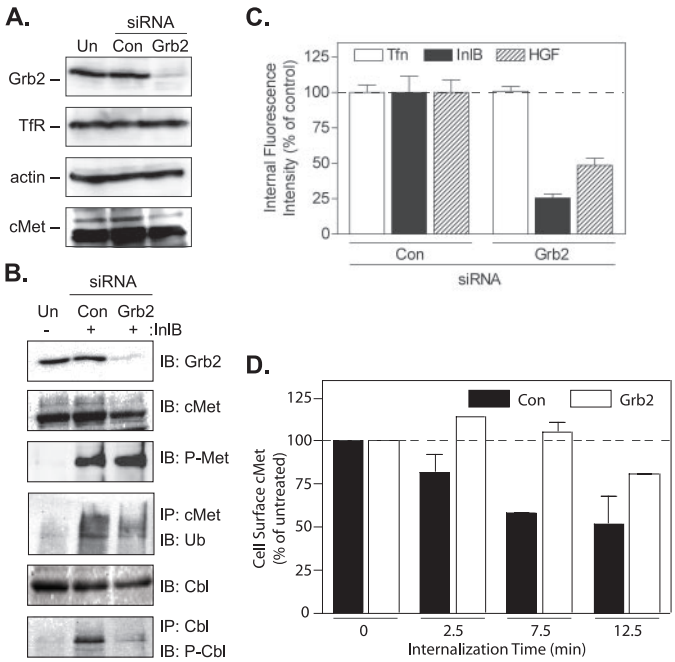

FIGURE 2. **siRNA-mediated Grb2 depletion inhibits InlB and HGF internalization.** *A*, lysates from mock-transfected (*Un*) T47D/cMet cells or cells transfected with control (*Con*) or Grb2 siRNAs were examined by Western analysis for Grb2, transferrin receptor (*TfR*), actin and cMet expression. *B*, mock-transfected T47D/cMet cells or cells transfected with the indicated siRNAs were treated for 10 min at 37 °C with (+) or without (−) 100 ng/ml InlB. Cell lysates were examined by Western analysis (*IB*) for expression of total protein (cMet, Grb2, Cbl), phosphorylation IP cMet and Cbl (PY20), and cMet ubiquitination (*Ub*). *C*, the relative amounts of internalized HGF, Alexa-InlB, and Tfn (10 min at 37 °C) were quantified by confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from four separate experiments and are expressed as a percentage of control values. *D*, T47D/cMet cells transfected with control or Grb2-specific siRNAs were treated with 100 ng/ml InlB for the indicated times at 37 °C, and surface cMet levels were quantified by flow cytometry analysis as described in the legend to Fig. 1*E*.

that endogenous Grb2 is required for cMet uptake in different cell types (supplemental Fig. S4). We confirmed the requirement for Grb2 in cMet internalization using flow cytometry. As expected, we observed higher levels of surface cMet in Grb2-depleted T47D/cMet cells relative to cells transiently transfected with a nonspecific control siRNA after receptor activation with InlB (Fig. 2*D*). Thus, loss of Grb2 led to a specific block in HGF- and InlB-induced cMet internalization and not a general inhibition in clathrin-mediated endocytosis.

Interactions Downstream of Grb2 Recruitment Mediate cMet Internalization—We used targeted dominant negative Grb2 mutants to determine whether the binding of Grb2 to cMet was important for receptor endocytosis. Grb2 consists of a central SH2 domain flanked by two SH3 domains. The central SH2 domain mediates Grb2 binding to phosphotyrosine 1356 in the multisubstrate docking site of activated cMet and to the tyrosine-phosphorylated adaptor protein Shc. The SH3 domains of Grb2 mediate its binding to critical signaling molecules including the adaptor Gab1 (56), cCbl (57, 58), and the large GTPase dynamin (59). The mutations P49L and G203R in the N- and C-terminal SH3 domains, respectively, (Fig. 3*A*), block these interactions in a double Grb2 mutant (Grb2-mSH3). If Grb2 is required for cMet internalization, we reasoned that in contrast to wt Grb2, expression of a mutant expressing only the SH2 domain of Grb2 (Grb2-SH2) would function as a dominant neg-

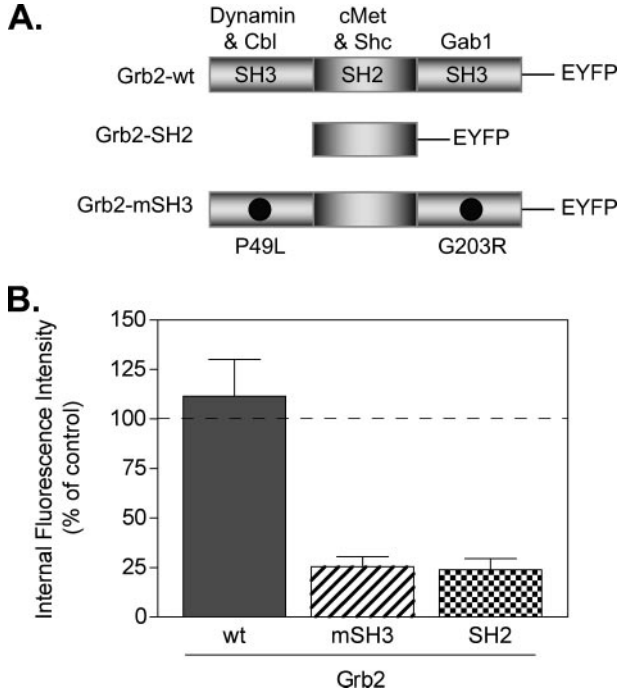

FIGURE 3. **Interactions downstream of Grb2 are involved in cMet internalization.** *A*, schematic of EYFP-tagged wt or mutant (*SH2*, *mSH3*) Grb2 and their binding properties. *B*, the relative amounts of internalized Alexa-InlB (10 min at 37 °C) in mock-transfected T47D/cMet cells and in cells expressing the indicated EYFP-tagged Grb2 proteins were quantified by confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from four separate experiments and are expressed as a percentage of control (mock-transfected) values.

ative mutant and block the binding of endogenous Grb2 to cMet. Conversely, the SH3 double mutant (Grb2-mSH3) would be unable to form productive complexes with downstream effectors and, hence, function to impede cMet internalization when expressed in cells. T47D/cMet cells were transfected with wt or mutant Grb2 (Grb2-SH2, Grb2-mSH3) labeled at their C termini with enhanced yellow fluorescent protein (EYFP) (Fig. 3*A*) to enable detection of the overexpressed proteins. The presence of EYFP has been shown not to interfere with the function of wt Grb2 or its mutants (22). Control Western analysis detected comparable levels of activated cMet in mock-transfected T47D/cMet cells and in cells expressing wt or mutant Grb2 (supplemental Fig. S5) in response to InlB. In contrast to wt Grb2, expression of Grb2-mSH3 and to a lesser extent Grb2-SH2 resulted in reduced extracellular signal-regulated kinase activation. The reduced activation of extracellular signal-regulated kinase observed in cells expressing Grb2-SH2 was consistent with the lower transfection rate routinely obtained using this construct (supplemental Fig. S5). The effect of wt and mutant Grb2 on Alexa-InlB uptake in T47D/cMet cells was quantified using confocal microscopy. After treatment with Alexa-InlB for 10 min at 37 °C to stimulate cMet internalization, comparable levels of internalized ligand were detected in control cells expressing EYFP only and in cells expressing wt Grb2 (Fig. 3*B* and supplemental Fig. S6). Conversely, an 84–90% decrease in the relative amount of internalized Alexa-InlB was detected in cells expressing Grb2-SH2 or Grb2-mSH3, indicating that functional interactions downstream of Grb2 binding are important for cMet internalization.

Cbl Ubiquitin Ligase Activity Is Required for cMet Internalization—Consistent with our data demonstrating a requirement for Grb2 in cMet internalization, we previously reported that receptor endocytosis is impaired in cells overexpressing dominant negative dynamin (3). However, it remains unclear which additional Grb2-mediated interactions are required for the internalization of soluble InlB·cMet or HGF·cMet complexes. Our initial studies focused on the E3 ubiquitin ligase Cbl for several reasons. Grb2-recuited Cbl ubiquitin ligase activity has been implicated in the clathrin-dependent internalization of EGFR in response to EGF (17–21). Grb2 has been shown to constitutively associate with the E3 ubiquitin ligase Cbl (60–62) and is recruited to cMet in response to receptor activation (4, 14). Overexpression of a Cbl mutant encompassing the N-terminal TKB and RING finger domains has been reported to decrease cMet internalization, possibly by blocking recruitment of CIN85-endophilin complexes to cMet (4). Although these studies demonstrated a role for Cbl in cMet internalization, they focused only on the adaptor functions of Cbl and did not examine the requirement for the ubiquitin ligase activity of Cbl for cMet internalization. Thus, the indirect recruitment of Cbl ubiquitin ligase activity through Grb2 could contribute to Grb2 function in cMet endocytosis.

We tested the idea that the ubiquitin ligase activity of Cbl was important for cMet internalization using confocal microscopy. T47D/cMet cells were transfected with wt Cbl or Cbl mutants defective in either ubiquitin ligase activity (70Z-Cbl) or binding to phosphotyrosine 1003 (Cbl-G306E) (Fig. 4*A*). Previous studies have demonstrated that Cbl binds cMet and induces its ubiquitination in response to HGF, whereas the ubiquitin ligase-deficient mutant Cbl-70Z interacts with cMet but is unable to induce receptor ubiquitination (4, 13, 14, 43). The cells were allowed to co internalize Tfn with Alexa-InlB or HGF for 10 min at 37 °C, and the relative amount of internalized ligand was examined by confocal microscopy (supplemental Fig. S7). cMet internalization was unaffected in mock-transfected control cells and in cells expressing wt Cbl or G306E, a mutant unable to bind RTKs directly. However, in cells overexpressing the ubiquitin ligase-deficient mutant 70Z-Cbl, internalized HGF and Alexa-InlB staining was reduced to 33.5 ± 2.4 and 50.4 ± 2.6% of control levels, respectively (Fig. 4*B*). To confirm the requirement of Cbl-mediated ubiquitination for cMet internalization, we took advantage of well characterized ubiquitin mutants that have altered conjugation and binding properties (Fig. 4*C*). Cbl functions to covalently attach monoubiquitin to substrate proteins via the C-terminal glycine residues of ubiquitin. The ubiquitin mutant UbR lacks these glycine residues important for substrate conjugation. However, UbR retains the ability to interact with intracellular proteins harboring ubiquitin binding domains, including the ubiquitin-interacting motif. Thus, when expressed in cells, UbR functions to block the interaction of ubiquitinated proteins with proteins harboring a ubiquitin-interacting motif (18). Using confocal microscopy, we examined the effect of transiently expressing mutant ubiquitin (UbR) on cMet internalization in T47D/cMet cells. UbR containing the double mutation L8A,I44A (UbR-L8A,I44A) that blocks the interaction of UbR with ubiquitin

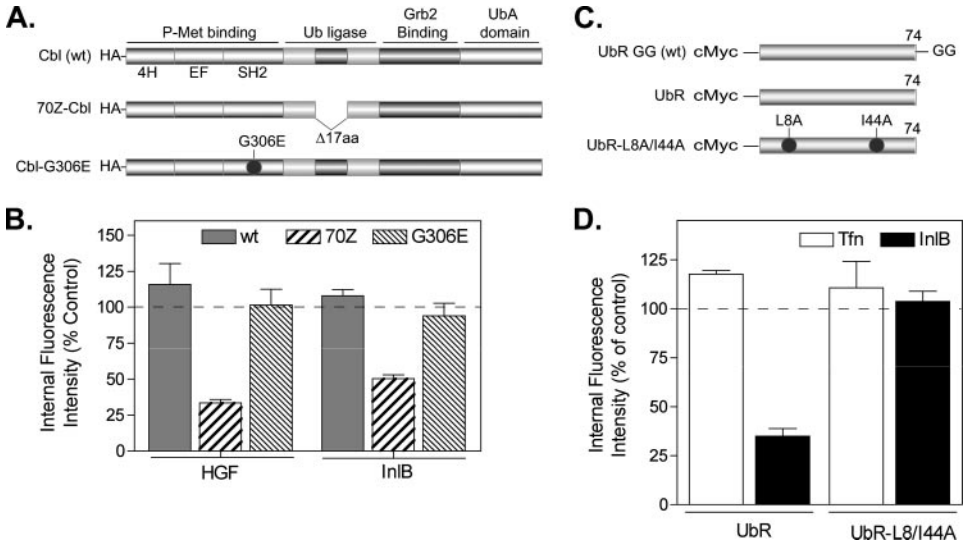

FIGURE 4. **Cbl ubiquitin ligase activity is involved in cMet internalization.** *A*, schematic of hemagglutinin-tagged wt or mutant Cbl (70Z, G306E) and their binding properties. *B*, mock-transfected T47D/cMet cells and T47D/cMet cells transiently expressing hemagglutinin-tagged wt or mutant Cbl were allowed to internalize Alexa-InlB or 100 ng/ml HGF for 10 min at 37 °C, and the relative amounts of internalized ligands were quantified by confocal microscopy. Internalized HGF was detected by staining cells with an anti-HGF antibody. Values represent the mean fluorescence intensity ± S.E. from four separate experiments and are expressed as a percentage of control (mock-transfected) values. *C*, schematic of cMyc-tagged wt ubiquitin (*UbR GG*), mutant ubiquitin (*UbR*) and the inactive variant UbR-L8A/I44A are shown. *D*, the relative amounts of internalized Tfn and InlB in transfected T47D/cMet cells expressing the indicated constructs were quantified using confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from four separate experiments and are expressed as a percentage of control (mock-transfected) values.

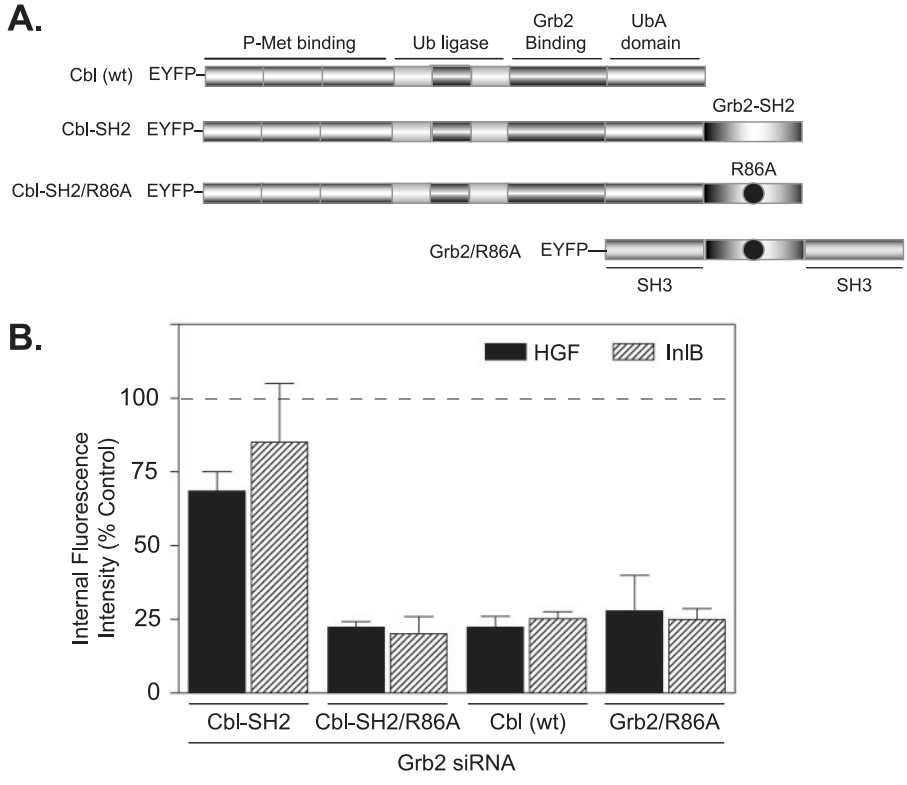

FIGURE 5. **Rescue of cMet internalization in Grb2-depleted cells using a Cbl-Grb2/SH2 chimeric protein.** *A*, schematic of EYFP-tagged mutant Grb2 (R86A) deficient in cMet binding, wild type Cbl (Cbl wt), Cbl fused to the SH2 (cMet binding) domain of Grb2 or a mutant Grb2-SH2 domain (SH2/R86A). *B*, T47D/cMet cells were co transfected with Grb2-specific siRNAs to deplete endogenous Grb2 and EYFP-tagged constructs as indicated. The relative amounts of internalized Alexa-InlB and HGF were quantified by confocal microscopy. Values represent the mean fluorescence intensity ± S.E. from 4 separate experiments and are expressed as a percentage of control (mock-transfected) values.

interacting motifs was used as a control (supplemental Fig. S8). As shown in Fig. 4*D*, expression of UbR reduced cMet internalization to 33.7 ± 3.4% relative to mock-transfected control cells or cells expressing the double mutant UbR-L8A,I44A. Tfn uptake was unaffected in cells expressing UbR or UbR-L8A,I44A. This is the first report indicating that the ubiquitin ligase activity of Cbl is important for cMet internalization in response to soluble InlB as well as HGF.

Recruitment of Cbl through the SH2 Domain of Grb2 Supports cMet Internalization—Our data indicating a role for Grb2 and the ubiquitin ligase activity of Cbl for the internalization of soluble ligand-cMet complexes suggested that the recruitment of a Grb2·Cbl complex could be important for cMet internalization. To test this we transiently transfected T47D/cMet siRNA-depleted of endogenous Grb2 with a chimeric protein in which wt Cbl was tagged with EYFP at its N terminus, and the SH2 domain of Grb2 was tagged at its C terminus (Cbl-SH2) (22). In the absence of endogenous Grb2, Cbl recruitment to the docking site of cMet would be predicted to occur via the Grb2-SH2 domain at the C terminus of the chimeric protein, thereby reversing the inhibition of cMet endocytosis imposed by Grb2 depletion. A second mutant chimeric protein deficient in cMet binding (Cbl-SH2/R86A) (22) was used as a control (Fig. 5*A*). In these studies, T47D/cMet cells were co transfected with Grb2-targeted siRNA and with Cbl-SH2, Cbl-SH2/R86A, or mock-transfected cells as a control. The cells were allowed to co internalize Alexa-InlB and HGF for 10 min at 37 °C. Given the low double transfection rates routinely observed using these constructs, we examined the relative amounts of internalized ligand using confocal microscopy (Fig. 5*B*). Internalized HGF was detected by co-staining with an antibody specific for HGF. Expression of the chimeric protein Cbl-SH2 in Grb2-depleted cells

caused increased uptake of Alexa-InlB and HGF in Grb2-depleted cells compared with Grb2-depleted cells expressing EYFP only. Conversely, expression of the chimeric protein deficient in cMet binding (Cbl-SH2/R86A) was not as effective at rescuing cMet internalization in Grb2-depleted cells. Similarly, overexpression of wt Cbl or Grb2/R86A (a Grb2 mutant deficient in cMet binding) did not reverse the inhibitory effect of siRNA-mediated Grb2 depletion on cMet internalization. Together, these data suggest that the major role of Grb2 in cMet endocytosis is to recruit Cbl to the receptor.

The Signaling Adaptor Gab1 Is Not Required for cMet Internalization—In addition to binding Cbl, Grb2 has been shown to bind Gab1, an adaptor protein that mediates almost all of the signaling cascades downstream from activated cMet including phosphatidylinositol 3-kinase, c-Jun N-terminal kinase, and extracellular signal-regulated kinase signaling (for review, see Ref. 63). Gab1 is recruited to cMet by two mechanisms. The primary mechanism involves the indirect recruitment of Gab1 to cMet via Grb2 (35, 38). In addition, Gab1 can bind directly to phosphotyrosine 1349 in the multisubstrate docking site of cMet (64). To examine the importance of Gab1 in cMet internalization, a Gab1-specific siRNA was used to deplete T47D/cMet cells of endogenous Gab1. Western analysis confirmed the specificity of the siRNA-mediated Gab1 knockdown in cells transfected with Gab1 but not a scrambled control (*Con*) siRNA (Fig. 6*A*). Comparable levels of Cbl, actin, Grb2, transferrin receptor, and cMet were detected in Gab1-depleted cells as well as mock-transfected cells and cells transfected with a control siRNA. Western analysis confirmed that siRNA depletion of Gab1 did not inhibit ligand-induced cMet phosphorylation and ubiquitination or Cbl phosphorylation (Fig. 6*B*). To examine the effect of Gab1 depletion on cMet internalization, siRNA-treated cells were incubated with InlB (100 ng/ml for 10 min at 37 °C) to activate cMet signaling, and the relative amounts of residual cell surface cMet were measured using flow cytometry (Fig. 6*C*). Comparable levels of cell surface cMet were detected in control and Gab1-depleted cells, indicating that siRNA-mediated knockdown of Gab1 did not inhibit cMet internalization in response to InlB. Confocal microscopy studies confirmed that cMet internalization in response to InlB or HGF was not decreased in Gab1 depleted T47D/cMet cells or cells transfected with a control siRNA (data not shown). Thus, loss of Gab1 does not block clathrin-mediated cMet internalization.

The Direct Binding of Grb2 to the Multisubstrate Docking Site of cMet Promotes Receptor Endocytosis—A similar role for Grb2 and Cbl in EGFR internalization has been reported (17, 18, 21, 22), suggesting a general role for these proteins in RTK endocytosis. However, it is unclear whether Grb2 functions by directly interacting with cMet at phosphotyrosine 1356. Alternatively, the ability of the adaptor Shc to recruit Grb2 indirectly to cMet (56) may underlie the requirement for Grb2 in cMet endocytosis. To distinguish between these possibilities, we generated a polyclonal cell line using cMet$^{-/-}$ null cells expressing moderate levels of the cMet mutant N1358H (NH-cMet). N1358H is a well characterized mutation that specifically interferes with the direct binding of Grb2 to cMet with no measurable effect on the recruitment of other signaling or adaptor

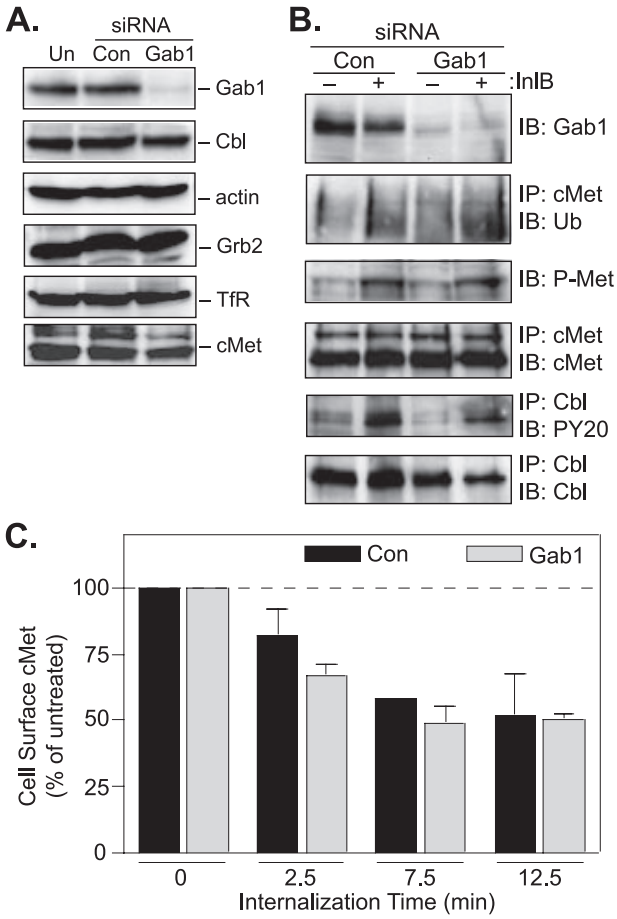

FIGURE 6. **siRNA-mediated Gab1 depletion does not inhibit InlB- and HGF-induced cMet internalization.** *A*, Western analysis of lysates from mock-transfected (*Un*) and T47D/cMet cells transfected with control (*Con*) or Gab1 siRNAs confirmed specific knock down of Gab1. *B*, T47D/cMet cells were transfected with the indicated siRNAs after treatment with (+) or without (−) 100 ng/ml InlB for 10 min at 37 °C. Equal amounts of lysates were examined by IP and Western analysis (*IB*) for total protein (cMet, Gab1, and Cbl), cMet phosphorylation (*P-Met*), ubiquitination (*Ub*), and Cbl phosphorylation (*PY20*) as indicated. *C*, T47D/cMet cells transfected with control or Gab1-specific siRNAs were treated with 100 ng/ml InlB for the indicated times at 37 °C, and surface cMet levels were quantified by flow cytometry analysis.

molecules including Shc (29, 65). Surface biotinylation studies confirmed that, like wt cMet, the mutant receptor NH-cMet was expressed at the cell surface (Fig. 7*A*). GST pulldown assays confirmed that recombinant Grb2 bound to wt cMet in response to InlB. Conversely, Grb2 binding to NH-cMet was inhibited under these conditions (Fig. 7*B*). cMet ubiquitination and Cbl phosphorylation in response to InlB was detectable in null cells expressing wt cMet but reduced in cells expressing the mutant NH-cMet (Fig. 7*C*). These data support the conclusion that the direct binding of Grb2 to the docking site of cMet is inhibited by the mutation N1358H. We next compared the internalization properties of NH-cMet with the wild type receptor. Using Alexa-InlB to stimulate cMet internalization (10 min, 37 °C), we confirmed that cMet internalization was reduced in cMet null cells stably expressing NH-cMet but not in cMet null cells stably expressing wt cMet by confocal microscopy (Fig. 7*D*). To confirm our confocal studies, we used flow cytometry analysis to compare the internalization properties of wt and NH-cMet. Cells were incu-

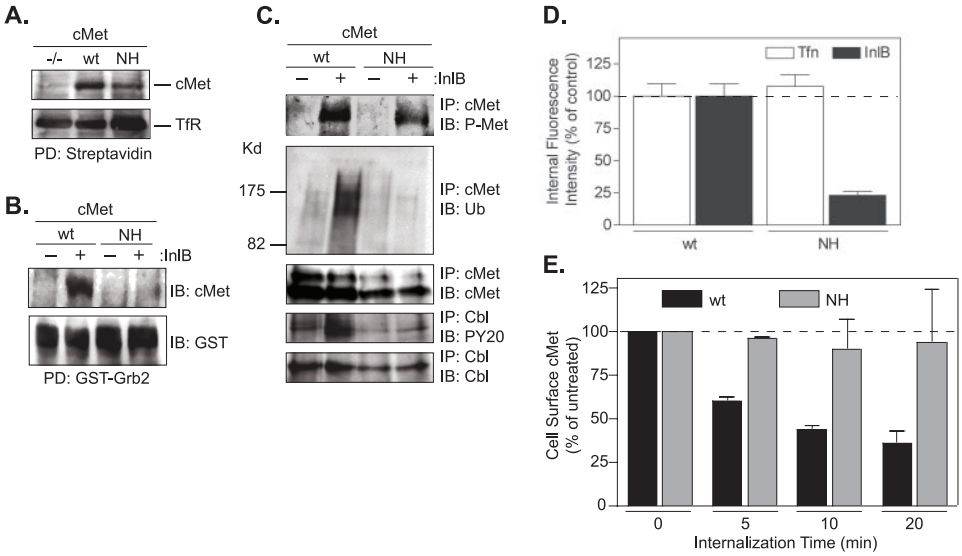

FIGURE 7. **Direct Grb2 recruitment is required for cMet internalization.** *A*, pulldown (*PD*) surface biotinylation assays confirmed that cMet null (−/−) cells stably express comparable levels of wt cMet or cMet-N1358H (*NH*), a mutant deficient in Grb2 binding, at their surface. *B*, cMet null (−/−) cells stably expressing wt or mutant cMet (*NH*) were treated with (+) or without (−) 100 ng/ml InlB for 10 min at 37 °C. Equal amounts of cell lysates were incubated with recombinant GST-Grb2 and examined by Western analysis (*IB*). Grb2 binding to mutant NH-cMet was inhibited in response to InlB treatment. *C*, total protein (cMet, Cbl), cMet ubiquitination (*Ub*), and phosphorylation (*P-Met*) and Cbl phosphorylation (*PY20*) in response to InlB (+) were examined in lysates prepared from cMet null (−/−) cells stably expressing wt or mutant cMet (*NH*) by IP and Western analysis. The positions of molecular mass markers are indicated (kDa). *D*, confocal microscopy detected reduced levels of internalized Alexa-InlB but not Tfn in cMet null (−/−) cells stably expressing mutant cMet (*NH*). Values represent the mean fluorescence intensity ± S.E. from four separate experiments and are expressed as a percentage of control (wt cMet) values. *E*, surface cMet levels on cells expressing wt or mutant (*NH*) cMet were determined after treatment with 100 ng/ml InlB for the indicated times at 37 °C and quantified by flow cytometry analysis. Results represented the mean fluorescent intensities normalized to untreated cells (0 min) from triplicate experiments. *Bars* represent the means for data across three experiments with S.E.

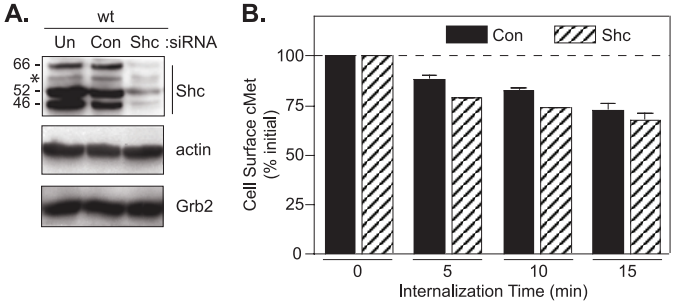

FIGURE 8. **cMet internalization does not involve Shc.** *A*, Western analysis of lysates from mock-transfected (*Un*) and cMet null cells expressing wt cMet transfected with control (*Con*) or Shc siRNAs for Shc, actin, and Grb2 confirmed specific knock down of the three family members of Shc. The *asterisk* indicates a nonspecific immunoreactive band. *B*, cMet$^{-/-}$ cells stably expressing wt cMet were transfected with control or Shc specific siRNAs then treated with 100 ng/ml InlB for the indicated times at 37 °C, and surface cMet levels were quantified by flow cytometry analysis. Results are graphed as a percentage of total surface cMet ± S.E. before internalization.

bated in media containing 100 ng/ml InlB for increasing periods of time at 37 °C and processed for flow cytometry. Under these conditions, the cell surface levels of NH-cMet remained unaltered over the course of the experiment. Conversely, cell surface levels of wt cMet decreased to 35.7 ± 7.6% after treatment with 100 ng/ml InlB for 20 min (Fig. 7*E*).

In contrast to our results above, Shc has been reported to regulate EGFR internalization (66), consistent with a role for the indirect recruitment of Grb2 for receptor internalization. To confirm that Shc is dispensable for cMet internalization, we used a siRNA approach in cMet$^{-/-}$ cells stably expressing wt cMet. Western analysis detected reduced levels of all three endogenous Shc proteins measuring 46, 52, and 66 kDa in cells transfected with Shc-siRNA but not in mock-transfected cells or cells transfected with a scrambled control siRNA (Fig. 8*A*). A nonspecific immunoreactive band was routinely detected using Shc-specific antibodies, consistent with previous reports using other cell lines (67). Comparable levels of actin and Grb2 were detected in Shc-depleted cells as well as mock-transfected cells and cells transfected with a control siRNA. We quantified the effects of siRNA-mediated Shc depletion on cMet internalization using flow cytometry analysis. Comparable levels of cell surface cMet were detected in cells transfected with control or Shc-siRNAs after treatment with 100 ng/ml InlB at all internalization times (Fig. 8*B*), indicating that cMet internalization is not dependent on the indirect recruitment of Grb2 through the adaptor Shc.

DISCUSSION

It was unclear whether the regulatory mechanisms for clathrin-mediated endocytosis vary for different RTK subfamilies. cMet and its oncogene TPR-Met are unique among RTKs in that two key tyrosine residues in their C-terminal multisubstrate docking sites mediate all of the biological and transforming activities of these receptors. In this study we examined the role of these tyrosines and their ability to bind specific signaling proteins for the internalization of the proto-oncogene cMet. Using variant forms of cMet with altered catalytic and binding properties, we demonstrate that clathrin-dependent cMet internalization is dependent on both the intrinsic kinase activity of the receptor and the recruitment of signaling molecules to phosphotyrosines 1349 and 1356 in the unique multisubstrate docking site of cMet. Depletion of Gab1 using siRNA did not inhibit cMet internalization. However, the levels of Gab1 were reduced but not abrogated in these studies, suggesting that low levels of Gab1 could be sufficient to allow cMet internalization. In a separate study we detected comparable levels of internalized cMet in mouse embryonic fibroblasts isolated from Gab1 null mice and a mouse embryonic fibroblast cell line isolated from their wild type litter mates stably expressing wt cMet.[3] Thus, we conclude that the recruitment of the signaling adaptor Gab1 was dispensable for cMet internalization. Our studies are the first to show a strict requirement for the ubiquitin ligase

[3] N. Li, M. Lorinczi, K. Ireton, and L. A. Elferink, unpublished data.

activity of Cbl for the internalization of complexes formed between cMet and two soluble receptor ligands, HGF and InlB. Consistent with a role for Cbl ubiquitin ligase activity, overexpression of an ubiquitin mutant that could not be conjugated to substrates yet retained the ability to bind to an ubiquitin interacting motif blocked ligand-induced cMet internalization. Together, our results broaden the roles of Grb2 and Cbl ubiquitin ligase activity to cMet internalization, a receptor-tyrosine kinase with unique structural and signaling characteristics.

A similar role for Grb2 has been reported for the EGFR. EGFR internalization through clathrin-coated pits was specifically blocked in Grb2-depleted cells (17). The block in EGFR internalization imposed by Grb2 depletion was rescued by transient expression of a Grb2·Cbl chimera (22). Overexpression of Grb2-SH3 mutants deficient in binding downstream signaling molecules inhibited the recruitment of EGFR to clathrin-coated pits (18) and prevented the translocation of Grb2 with activated EGFR into endocytic structures, presumably endocytic transport vesicles (48). In a separate study, overexpression of Grb2 with inactivating point mutations in the SH3 domains inhibited EGF-induced coated pit formation, with negligible effects on the downstream activation of mitogen-activated protein kinase or phosphatidylinositol 3-kinase (19). Our studies showing that Grb2-mediated internalization of cMet was dependent on the ubiquitin ligase activity of Cbl are consistent with several models for EGFR internalization in which the Grb2·Cbl complex likely functions to couple distinct stages of RTK endocytosis (20, 22, 68).

The multiadaptor Gab1 plays a critical role in cMet signaling by providing a scaffold for the simultaneous activation of several downstream signaling cascades including the activation of phosphatidylinositol 3-kinase, c-Jun N-terminal kinase, and extracellular signal-regulated kinase signaling (for review, see Ref. 63). Our data showing that the recruitment of Gab1 to cMet is dispensable for receptor internalization are consistent with our previous report that phosphatidylinositol 3-kinase signaling was not required for cMet endocytosis (3). cMet internalization was unaffected by overexpression of a dominant negative mutant for the p85 subunit of phosphatidylinositol 3-kinase. Similarly, no differences in the internalization properties of cMet were observed in T47D/cMet cells treated with the phosphatidylinositol 3-kinase inhibitors LY294002 or wortmannin (3). Thus, although Gab1 likely regulates the intensity and duration of cMet signaling, this does not appear to occur at the level of receptor internalization.

The recruitment of Grb2 directly or indirectly via the adaptor Shc to phosphotyrosine 1356 of cMet is essential for cell transformation and experimental metastases in response to oncogenic Met (69). The ability of Shc to form a stable complex with Grb2 in response to HGF (56) and EGF (70, 71) suggested that Grb2 recruited indirectly via Shc could regulate RTK endocytosis. Our data showing that cMet internalization was impaired by the N1358H mutation suggested that the direct recruitment of Grb2 to cMet rather than Grb2 recruited indirectly through Shc mediates cMet internalization, since Shc binding is unaffected by the N1358H mutation (29, 65). Consistent with this idea, siRNA-mediated Shc depletion did not interfere with cMet internalization through clathrin-coated pits. Thus, in light of our observations, we propose that Grb2 bound directly to cMet is indispensable for receptor endocytosis. In contrast to cMet, Shc has been reported to play a role in EGF-induced EGFR internalization (66, 72). EGF has been shown to promote the formation of a complex between wild type Shc and the adapter complex AP2 that is recruited to phosphotyrosine 1148 of the EGFR. Consistent with a role for Shc in EGFR endocytosis, the internalization rate constant of an EGFR truncation mutant lacking the region encompassing phosphotyrosine 1148 was significantly slower than that observed for the wild type receptor (73). Thus, the mode of Grb2 recruitment appears to diverge for cMet *versus* EGFR internalization, suggesting that distinct subsets of Grb2-mediated interactions are involved in the clathrin-dependent internalization of different RTKs.

The precise mechanism by which Cbl regulates cMet internalization and RTK endocytosis in general remains controversial. Cbl can be viewed as a bimodal molecule that functions as an adaptor protein that physically links Cbl/cMet complexes to the endocytic machinery (4) and as an E3 ubiquitin ligase to ubiquitinate cMet (13, 14) and its associated endocytic components. Although Grb2 and Cbl ubiquitin ligase activity was recently reported to be important for *L. monocytogenes* infection (5), these studies focused on the internalization of *Listeria* expressing surface anchored InlB and not soluble InlB. Moreover, these studies did not examine cMet internalization directly but instead monitored bacterial engulfment. A role for Cbl as an adaptor that links HGF-activated cMet to the endophillin-CIN85 complex (4) has been clearly demonstrated. In these studies inhibition of complex formation using dominant interfering forms of endophilin, CIN85, or Cbl was sufficient to inhibit cMet internalization and enhance cMet signaling. However, these studies did not address the requirement for Cbl ubiquitin ligase activity in this event or the mode of Cbl recruitment to cMet. Our studies showing that expression of a Cbl mutant deficient in ubiquitin ligase activity blocks cMet internalization and that a Grb2·Cbl chimera rescues receptor endocytosis in cells depleted of endogenous Grb2 are the first to show a definitive role for Cbl ubiquitin ligase activity in this process. Moreover, our studies indicate that Grb2 functions as a critical intermediary for Cbl ubiquitin ligase activity in the internalization of soluble InlB·cMet and HGF·cMet complexes. Cbl has been shown to mediate ubiquitination of cMet in response to HGF (43) in a process that involves the phosphotyrosine 1003 residue in the juxtamembrane region of cMet (13, 14, 42, 74). The observation that internalization of an ubiquitination-deficient Y1003F-cMet mutant is unaffected implies that receptor ubiquitination may not be a critical determinant for receptor internalization. Rather, Cbl-mediated ubiquitination of cMet via phosphotyrosine 1003 is important for degradation of the internalized receptor (13). Because the Y1003F-cMet was weakly ubiquitinated, we cannot rule out the possibility that different levels of, or sites of cMet ubiquitination contribute to distinct steps for cMet endocytic trafficking. Alternatively, clathrin-dependent internalization and receptor degradation may be regulated by the indirect recruitment of Cbl via Grb2 to phosphotyrosine 1356 *versus* Cbl recruited directly to phosphotyrosine 1003.

In conclusion our data demonstrate a strict requirement for a specific subset of Grb2-mediated interactions for cMet internalization from the cell surface. The direct binding of Grb2 to cMet is essential for receptor internalization. Moreover, endocytosis of soluble InlB·cMet and HGF·cMet complexes is dependent on the E3 ubiquitin ligase activity of Cbl and does not involve the Grb2 binding partner Gab1. Thus, Grb2 functions as an important intermediary for linking ligand-activated cMet with the ubiquitin ligase activity of Cbl for receptor internalization via clathrin-coated pits.

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Specific Grb2-mediated Interactions Regulate Clathrin-dependent Endocytosis of the cMet-tyrosine Kinase

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