Shedding-generated Met receptor fragments can be routed to either the proteasomal or the lysosomal degradation pathway.

Shedding-Generated Met Receptor Fragments can be Routed to Either the Proteasomal or the Lysosomal Degradation Pathway

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Abstract

The receptor tyrosine kinase Met and its ligand, the hepatocyte growth factor/scatter factor, are essential for embryonic development, whereas deregulation of Met signaling pathways is associated with tumorigenesis and metastasis. The presenilin-regulated intramembrane proteolysis (PS-RIP) is involved in ligand-independent downregulation of Met. This proteolytic process involves shedding of the Met extracellular domain followed by γ-secretase cleavage, generating labile intracellular fragments degraded by the proteasome. We demonstrate here that upon shedding both generated Met N- and C-terminal fragments are degraded directly in the lysosome, with C-terminal fragments escaping γ-secretase cleavage. PS-RIP and lysosomal degradation are complementary, because their simultaneous inhibition induces synergistic accumulation of fragments. Met N-terminal fragments associate with the high-affinity domain of HGF/SF, confirming its decoy activity which could be reduced through their routing to the lysosome at the expense of extracellular release. Finally, the DN30 monoclonal antibody inducing Met shedding promotes receptor degradation through induction of both PS-RIP and the lysosomal pathway. Thus, we demonstrate that Met shedding initiates a novel lysosomal degradation which participates to ligand-independent downregulation of the receptor.

Met is a receptor tyrosine kinase (RTK) expressed predominantly in cells of epithelial origin. It is activated by its stromal ligand, the hepatocyte growth factor/scatter factor (HGF/SF) [1]. HGF/SF and Met are essential for embryonic development, since knock-out of either one affects placenta, liver, muscle and neuron formation [2-5]. In adults, the HGF/SF-Met pair is involved in physiological processes like mammary gland development [6], wound healing and kidney or liver regeneration [7, 8]. *In vitro*, ligand-activated Met stimulates proliferation, scattering, invasion and morphogenesis of epithelial cells; acts as an angiogenic factor and has chemoattractant and neurotrophic activities [1].

Upon ligand binding and subsequent dimerization of Met, several tyrosine residues in the intracellular portion become phosphorylated, allowing recruitment of cytoplasmic proteins involved in activating multiple intracellular signaling pathways [9, 10].

Aberrant Met and HGF/SF signaling are involved in promoting tumorigenesis and metastasis. A direct link between Met and cancer has been evidenced by characterization of receptor activating mutations in the germline of patients affected by hereditary papillary renal carcinoma [11]. In a significant number of human cancers, HGF/SF and Met are

overexpressed [1]. Ligand-independent activation of the Met tyrosine kinase can also occur. It is typically observed in cells expressing high levels of receptor and leads to spontaneous dimerization and subsequent activation [12].

Downregulation of the HGF/SF-activated receptor is an essential negative regulatory mechanism preventing receptor oversignaling. Upon Met activation, the juxtamembrane tyrosine residue 1003 is phosphorylated, allowing interaction with the E3 ubiquitin ligase CBL [13]. This recruitment leads to ubiquitination of the receptor and causes the intracellular trafficking of Met and its degradation in the lysosomal compartment [14-16]. Uncoupling of Met- from Cbl-mediated ubiquitination, either through loss of the juxtamembrane domain or by mutation of the tyrosine residue acting as a Cbl docking site, leads to cell transformation [13, 17, 18].

We previously demonstrated that in the absence of ligand stimulation, Met is downregulated following proteolytic cleavages. Under apoptotic conditions, Met is cleaved by caspases, which separate the extracellular ligand-binding domain from the intracellular kinase domain; the generated cytoplasmic fragment (p40 Met) is involved in apoptosis amplification [19-22]. We have also demonstrated that Met is processed by PS-RIP (presenilin-regulated intramembrane proteolysis) [23]. Met is cleaved within its extracellular juxtamembrane domain by membrane metalloproteases. Silencing of ADAM10 inhibits constitutive and stimulated-shedding induced by the anti-Met antibody DN30 [24, 25]. In addition, silencing of ADAM17 was reported to inhibit partially Met shedding, suggesting involvement of several membrane metalloproteases [23]. This cleavage generates soluble Met N-terminal fragments (Met-NTF), which are shed into the extracellular space, and membrane-anchored Met C-terminal fragments (Met-CTF) [15, 26-28]. The latter are in turn cleaved at the membrane by the γ-secretase complex, of which presenilin is the catalytic subunit. The generated remnant intracellular domains of Met (Met-ICD) are released into the cytosol and degraded by the proteasome [23]. We have demonstrated that constitutive cleavage by PS-RIP and further degradation of the Met intracellular domains contribute to decreasing the half-life of the receptor and to preventing its accumulation in the membrane [23].

Numerous membrane receptors are cleaved by PS-RIP, including at least six RTKs [29]. In most cases, these sequential cleavages downregulate RTK expression, through generation of a labile intracellular fragment degraded by the proteasome. This has been evidenced for the EphB2, IGF1, Tie1 and CSF receptors [30-33]. In addition, intracellular fragments generated from ErbB4 and Ryk can translocate to the nucleus and bind to transcriptional repressors or activators to regulate gene expression [34-36].

We demonstrate that the fragments generated by ectodomain shedding can escape γ-secretase processing through direct lysosomal degradation. This pathway constitutes a novel ligand-independent degradation mechanism participating in downregulation of Met.

Results

Inhibition of lysosomal degradation leads to generation of Met-CTF

Although the lysosomal degradation of full length Met is well described, the involvement of this degradation pathway in elimination of the recently discovered Met intracellular fragments has not been investigated. For this purpose, the human mammary epithelial cell line MCF-10A were treated or not with bafilomycin A1, an H^+−pump (V-ATPase) inhibitor well known to prevent lysosomal degradation, in the presence or absence of HGF/SF for different times [37]. Met receptor expression was evaluated on western blots probed with an antibody against its kinase domain. As expected, HGF/SF stimulation induced Met phosphorylation, followed by its degradation, more than half of the receptor being degraded upon stimulation for 2 h (Figure 1A). Bafilomycin A1 inhibited full length Met degradation, confirming the lysosome-dependent downregulation of Met upon ligand stimulation [14]. Interestingly, we observed additional fragments in bafilomycin-A1-treated samples, ranging in size from 55 to 60 kDa, whether HGF/SF was present or not (Figure 1A). This suggests that Met-CTFs are degraded by the lysosome, independently of ligand stimulation.

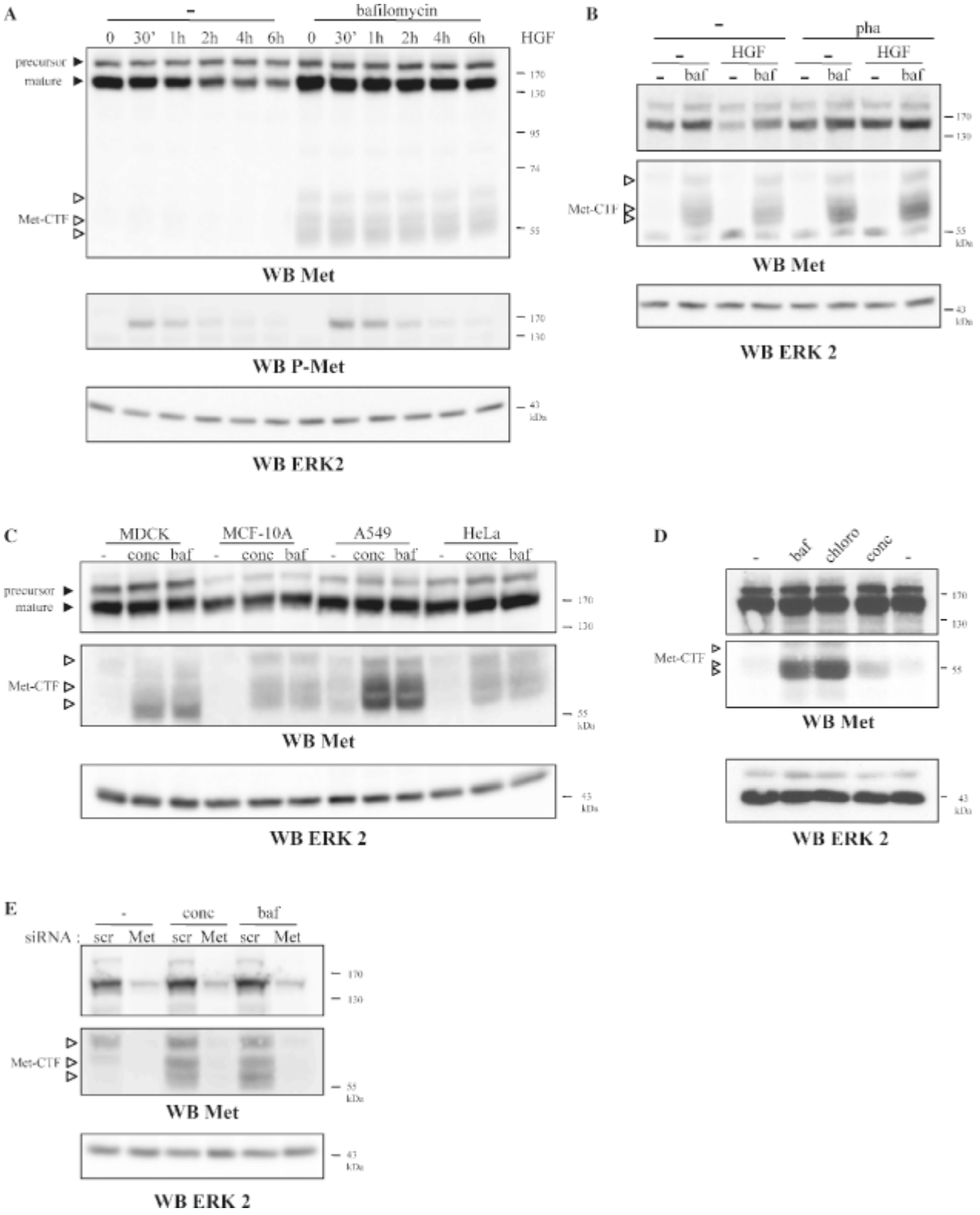

Figure 1. Stabilization of Met C-terminal fragments by H+−pump inhibitor.A) MCF-10A cells were treated overnight with 30 ng/mL HGF/SF in the presence and absence of 5 nM bafilomycin A1 (baf). B) MCF-10A cells were treated for 16 h with 300 nM PHA665752 a Met inhibitor (pha) and/or 5 nMbafilomcin A1 and/or for 8 h with 30 ng/mL HGF. C) MDCK, MCF-10A, A549, and HeLa cells were treated for 16 h with 10 nM concanamycin A (conc) and 5 nM bafilomycin A1. D) MDCK cells were treated for 16 h with 5 nM bafilomycin A1 and 50 µM chloroquine (chloro) and for 5 h with 10 nM concanamycin. E) MCF-10A cells were transfected with scrambled or targeting Met siRNA. The following day the cells were treated for 16 h with 10 nM concanamycin A or 5 nM bafilomycin A1. For each treatment, the same amount of protein was resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies against phosphorylated residues of the Met kinase domain, antibodies against the kinase domain of Met, and antibodies

against ERK2 to assess loading. The positions of prestained molecular weight markers are indicated. Arrows indicate the positions of precursor and mature full-length Met and C-terminal Met fragments (Met-CTF).

HGF/SF-stimulated Met degradation was prevented by a selective Met kinase inhibitor (Figure 1B), demonstrating the requirement of the Met kinase activity for ligand-dependent degradation. In contrast, Met-CTFs were detected in bafilomycin-A1-treated samples whether HGF/SF or the Met kinase inhibitor was present or not. Similar Met-CTFs were stabilized with concanamycin A, another H+−pump inhibitor, in various epithelial cell lines, including the kidney epithelial cell line MDCK, the mammary cell line MCF-10A, the lung tumor cell line A549, and the cervical tumor cell line HeLa (Figure 1C). In addition, chloroquine, which prevents multi-vesicular bodies and lysosome fusion, stabilized also the Met-CTFs in MDCK cells (Figure 1D). When Met expression was silenced in MCF-10A cells with a specific siRNA, the band corresponding to full-length Met was markedly reduced and no bands appeared in the 55- to 60-kDa range. This confirms that the fragments stabilized by lysosome inhibitors are indeed fragments of Met (Figure 1E).

Altogether, these results indicate that the lysosomal compartment is involved not only in ligand-dependent downregulation of Met, but also in the degradation of Met-CTFs. In contrast to the previously described lysosomal degradation of full-length Met, the generation and degradation of Met-CTFs does not depend on ligand stimulation or Met kinase activity.

Met-CTFs are generated by shedding but escape γ-secretase processing

The molecular weights of the Met-CTFs stabilized by lysosome inhibitors suggest the release of the extracellular domain of Met and are reminiscent of those generated upon Met shedding [23]. To determine whether these fragments are generated upon cleavage of Met within the extracellular juxtamembrane region, we looked for Met-CTFs in cells expressing either an uncleavable chimeric receptor (uncleavable TRK-Met, in which the entire extracellular domain of Met is replaced with the extracellular domain of the TRKA receptor), or a cleavable chimera in which the first 50 juxtamembrane extracellular amino acids of Met are present (cleavable TRK-Met) (Figure 2A) [23]. As previously shown, because of its high rate of shedding, the full-length form of cleavable TRK-Met was found at a lower level than the uncleavable TRK-Met (Figure 2B). As expected, treatment with the γ-secretase inhibitor Compound E stabilized Met-CTFs only in cells expressing the cleavable TRK-Met. Treatment with lysosomal inhibitors likewise stabilized Met-CTFs in cells expressing the cleavable TRK-Met but not in those expressing the uncleavable chimera (Figure 2B). This demonstrates that shedding of the extracellular domain is a prerequisite to generation of lysosomally degraded fragments. It is worth noticing that in cells expressing uncleavable TRK-Met, treatment with bafilomycin increased the full length Met level. We previously showed that uncleavable chimeras are constitutively activated, suggesting that lysosome inhibition could prevent degradation of the activated receptors [23]. Treatment with Met kinase inhibitor stabilized also the full length uncleavable TRK-Met confirming degradation of these kinase active receptors (Figure 2C).

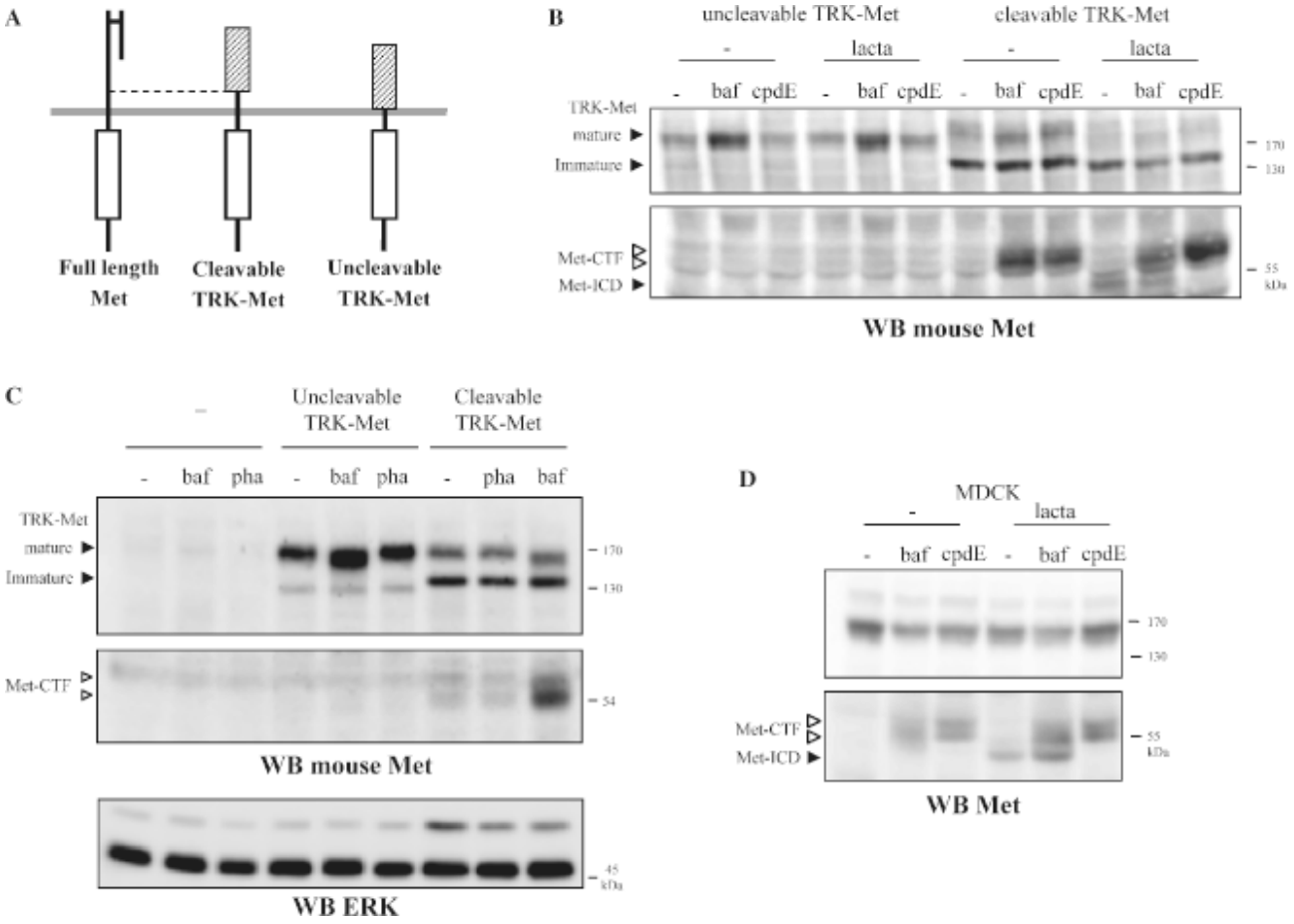

Figure 2. Generation of Met C-terminal fragments by lysosome and γ-secretase inhibition. A) Schematic representation of full-length Met, uncleavable TRK-Met (consisting of the extracellular portion of TRKA fused to the transmembrane and intracellular domains of Met), and cleavable TRK-Met, possessing 50 additional amino acids of the extracellular juxtamembrane domain of Met. B) MDCK cells expressing cleavable or uncleavable TRK-Met chimeras were treated for 5 h with 10 μM lactacystin, for 16 h with 5 nM bafilomycin A1, or for 16 h with 1 μM compound E. C) MDCK cells expressing TRK-Met chimeras were treated for 16 h with 5 nMbafilomycin A1 or 300 nM PHA665752. D) MDCK cells were treated for 5 h with 10 μM lactacystin and for 16 h with 5 nM bafilomycin A1 or for 16 h with 1 μM compound E. For each treatment, the same amount of protein was resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies directed against the C-terminal region of mouse Met (B and C), the kinase domain of Met (D) and against ERK2. Arrows indicate positions of the Met C-terminal fragments (Met-CTF), the Met intracellular domain (Met-ICD) and mature and immature TRK-Met chimeras.

As previously shown, treatment of epithelial cells with proteasome inhibitor lactacystin stabilized the 50 kDa Met intracellular domain (Met-ICD) [23]. Further treatment with γ-secretase inhibitors stabilized the Met-CTFs at about 55 kDa, at the expense of Met-ICD, demonstrating sequential cleavage of Met by PS-RIP and further degradation by the proteasome (Figure 2D) [23]. In contrast, co-treatment of the cells with lysosome and proteasome inhibitors stabilized both Met-ICD and Met-CTF, demonstrating that the Met-CTFs degraded by the lysosome escape γ-secretase cleavage. Similar results were obtained with MDCK cells expressing cleavable TRK-Met (Figure 2B).

We next performed immuno-fluorescence to reveal the subcellular location of the Met fragments stabilized with either γ-secretase or lysosome inhibitors. Without treatment, immunofluorescence staining with antibody against the kinase domain of Met revealed receptor mainly at the plasma membrane, with weak perinuclear staining likely corresponding to neo-synthesized Met in the endoplasmic reticulum (Figure 3). Inhibition of γ-secretase led to a slight increase in plasma membrane staining, confirming that the Met-CTFs stabilized under these conditions are anchored to the cytoplasmic side of the membrane. In contrast, inhibition of lysosomal degradation led to detection of Met in intra-cytoplasmic vesicles. The intracellular Met staining co-localized with anti-Lamp-1 staining, a lysosomal marker (Figure 3). These results confirm that the Met-CTFs stabilized by γ-secretase and lysosome inhibitors display different localizations: a membrane

localization in the first case and a lysosomal localization in the second. Similar results were obtained with an anti-Met antibody directed against the N-terminal region of Met, suggesting that this region is also degraded through the lysosomal pathway (data not shown).

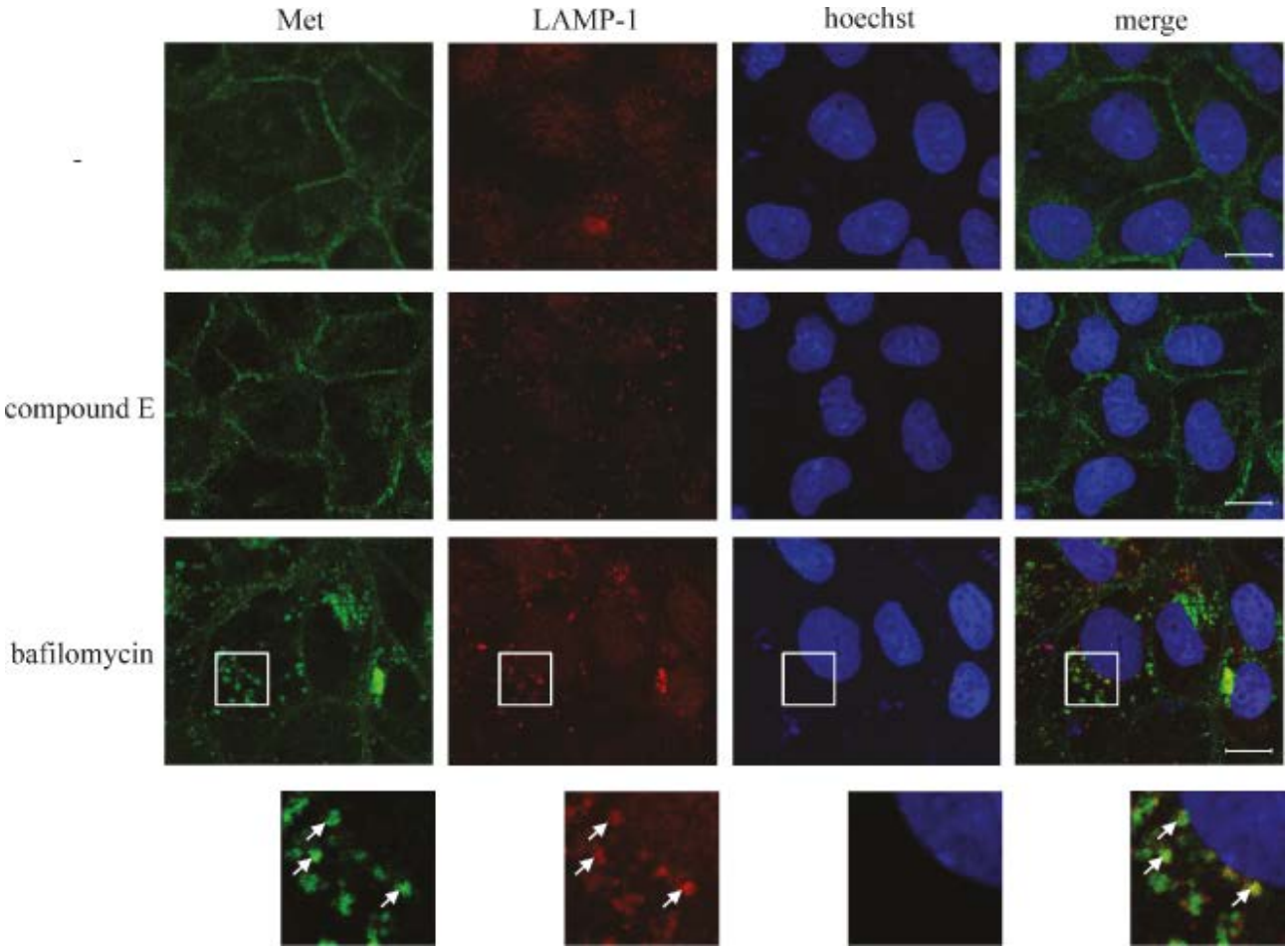

Figure 3. Membrane or lysosomal localization of Met C-Terminal fragments. MDCK cells were treated for 16 h with 1 μM compound E (cpdE) or 5 nM bafilomycin A1 and were stained with an antibody against the kinase domain of Met (green) and a rabbit antibody against LAMP-1 antibody (red). The nuclei were stained with Hoëchst (blue) and overlayed photogaphs are shown (merge). The images, taken at 63× magnification with a confocal microscope, are representative of experiments performed in triplicate and the white arrows indicate positions of colocalized staining. Scale bars = 10 μm.

Altogether, our results suggest that the Met-CTFs generated upon shedding of the N-terminal part of the receptor can be degraded by two processes: (i) direct lysosomal degradation and (ii) further cleavage by γ-secretase, generating Met-ICD, which in turn is degraded by the proteasome.

The lysosomal and γ-secretase degradation pathways cooperate in ligand-independent Met degradation

As Met-CTFs can be degraded either in the lysosome or, upon γ-secretase cleavage, by the proteasome, we assessed whether these degradation pathways might cooperate. Met-CTF stabilization was evaluated in MDCK, HeLa, MCF-10A and MDA-MB 231 cells treated with a lysosome inhibitor bafilomycin , the γ-secretase inhibitor compound E, or both. In MDCK and HeLa cells, similar amounts of Met-CTFs were stabilized by treatment with either inhibitor, while co-treatment induced synergistic stabilization (Figure 4A). Raising concentrations of compound E and two other γ-secretase inhibitors, L-685,458 and DAPT, induced similar synergistic stabilization of Met-CTF, assessing that this cooperation is not the consequence of inefficient γ-secretase inhibition (Figure S1). Similar cooperation was observed in MDCK cells expressing cleavable TRK-Met, but no Met-CTFs were detected in cells expressing uncleavable TRK-Met (Figure 4B). This indicates that both pathways cooperate to degrade Met-CTFs in these epithelial cells.

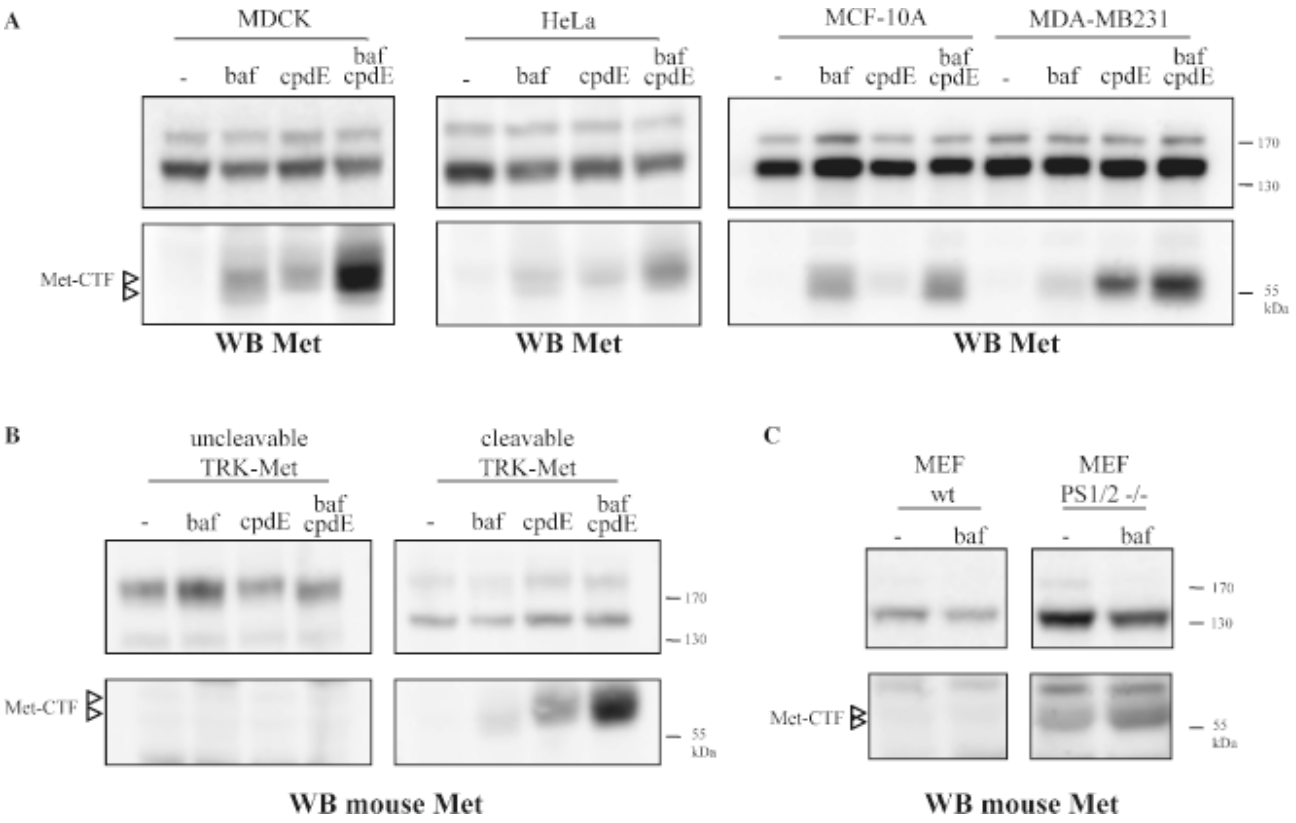

Figure 4. Stabilization of Met C-terminal fragments by inhibition of both the γ-secretase and the lysosomal degradation pathway. A) MDCK, HeLa and MCF-10A cells were treated for 16 h with 5 nM bafilomycin A1 and/or 1 μM compound E. B) MDCK cells expressing cleavable or uncleavable TRK-Met chimeras were treated for 16 h with 5 nM bafilomycin A1 and/or 1 μM compound E. C) Wild-type and presenilin-1- and presenilin-2-deficient MEFs (MEFs WT or PS1/2 −/−) were treated for 16 h with 5 nM bafilomycin A1 and/or 1 μM compound E. For each treatment, the same amount of proteins was resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies directed against the kinase domain of Met (A) and the C-terminal end of mouse Met (B and C). Arrows indicate positions of Met C-terminal fragments (Met-CTF).

In MCF-10A cells, the lysosome inhibitor efficiently induced Met-CTF stabilization, while γ-secretase inhibition led to minor stabilization (Figure 4A). Inversely, in MDA-MB231 cells, the lysosome inhibitor induced minor Met-CTF stabilization, while γ-secretase inhibition led to their efficient stabilization (Figure 4A, right panels). In mouse embryonic fibroblasts (MEF), the lysosome inhibitor did not induce fragment stabilization, but Met-CTFs were readily detected in presenilin-1- and presenilin-2-deficient MEFs, where the γ-secretase complex is not functional (Figure 4C). In these cells, however, further treatment with the lysosome inhibitor increased the generation of Met-CTFs, providing genetic evidence for cooperation between the γ-secretase and lysosomal pathways.

Our results thus demonstrate that both pathways contribute to degrade C-terminal Met receptor fragments and that the routing of these fragments varies according to the cell type. In some cell types, targeting to the lysosome appears to predominate.

When released in the medium, Met-NTFs are able to interact with the high-affinity domain of HGF

It is well known that the shedding process involves the generation of Met-NTFs of about 100 kDa which, being released into the extracellular medium, are undetectable in the intracellular compartment. As degradation of the CTF within the lysosome involved their previous internalization, we search to know whether the N-terminal counterparts could also be degraded in this compartment. This was evaluated in MDA-MB231 cells in which Met-CTFs are predominantly cleaved by γ-secretase, in MCF-10A cells in which they are mainly degraded in the lysosome, and in HeLa cells in which both degradation pathways are involved (Figure 4A). Upon bafilomycin treatment, Met-NTF, revealed with an antibody raised against the luminal domain of the Met β-chain, were stabilized in the three cell lines with a stronger detection in MCF10A and HeLa cells (Figure 5A). In untreated or γ-secretase-inhibitor-treated cells, Met-NTFs were not detected in the lysate.

As the Met-NTF can be degraded within the lysosome, we hypothesized that this routing occurs at the expense of their extracellular release. Accordingly, detection of the Met-NTF in the medium indicates that they are more released in the conditioned medium from MDA-MB231 than those from MCF10A and HeLa cells (Figure 5B). We previously demonstrated that the shedding process generates Met-NTFss, released into the medium, and a membrane-anchored Met-CTF, further cleaved by γ-secretase [23]. We now demonstrate that this initial cleavage of Met can also generate both Met-NTFs- and Met-CTFs that are directly degraded in the lysosomal compartment.

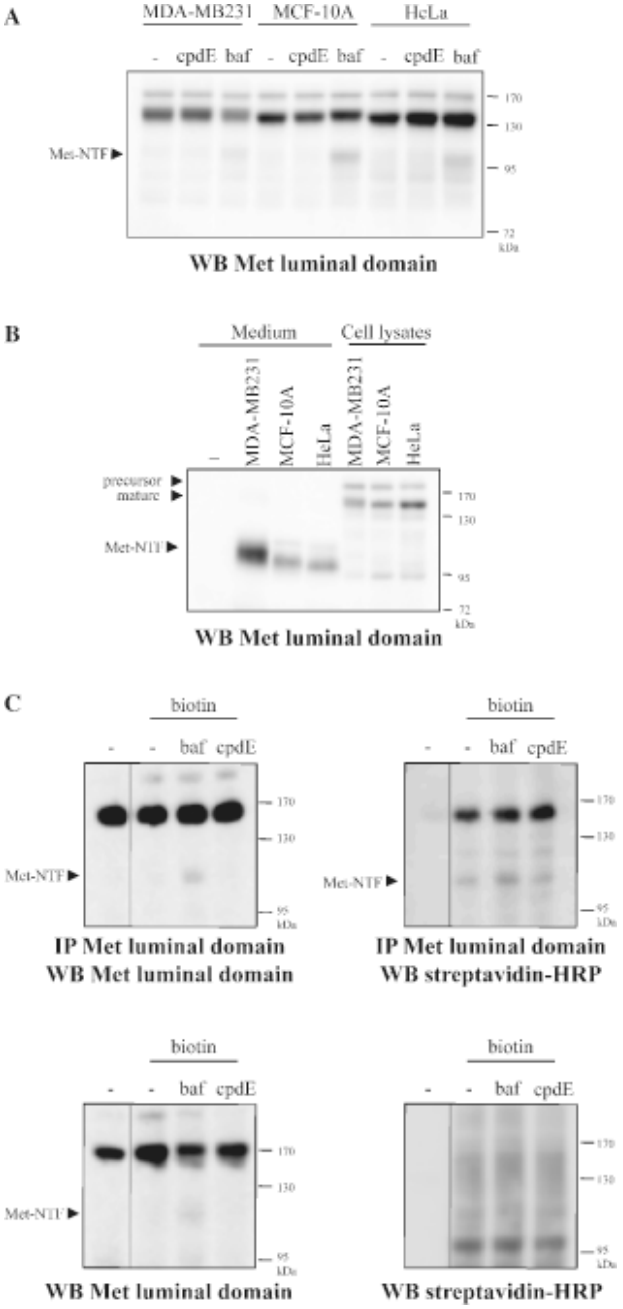

Figure 5. Consequence of Met routing to degradation pathways on the extracellular release of the Met N-terminal fragments. A) MDA-MB231, MCF-10A and HeLa cells were treated for 16 h with 5 nM bafilomycin A1 and/or 1 μM compound E. B) Conditioned serum-free medium of untreated MDA-MB231, MCF-10A and HeLa cells were collected and the corresponding whole-cell lysate were prepared. A and B) Proteins were resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies directed against the luminal region of the Met β-chain (luminal domain). C) After biotinylation of membrane proteins, HeLa cells were treated for 4 h with 5 nM bafilomycin A1 or 1 μM compound E while control cells were neither biotinylated nor treated. The cells were then lysed and Met was immunoprecipitated with antibody against the Met N-terminal region. The proteins present in whole-cell lysates or immunoprecipitates were resolved by 10% SDS-PAGE and analyzed by western blotting with streptavidin-HRP and with antibodies directed against the N-terminal region of Met. Arrows indicate the positions of the N-terminal fragments of Met (Met-NTF).

To make sure that the Met-NTFs were generated from the full-length receptor expressed at the cell surface, we stained extracellular proteins of HeLa cells with an impermeable biotinylating reagent. The Met receptor was then immunoprecipitated from the cell lysate and revealed either with antibody against the N-terminal domain of Met or with streptavidin-HRP, which recognizes biotinylated proteins. As expected, in both whole-cell lysates and immunoprecipitates, western blotting with anti-Met antibody revealed full-length forms of the receptor in all the samples and Met-NTFs only in cells treated with bafilomycin A1 (Figure 5A,C). In the immunoprecipitates, streptavidin-HRP detection revealed the full-length mature form of Met, consistently with staining of membrane-expressed proteins. In addition, a 100-kDa fragment was observed under all conditions, the corresponding band being more pronounced after bafilomycin A1 treatment. This confirms that the Met-NTFs are generated from membrane-anchored Met receptor (Figure 3B). The detection of biotinylated Met-NTFs even without lysosome inhibition suggests that biotinylation could inhibit lysosomal degradation.

We demonstrated that shedding-generated Met-NTF can be degraded in the lysosome instead of release in the extracellular medium. Interestingly, these fragments have been proposed to act as decoy for HGF/SF. However, their direct interaction with HGF/SF is still controversial [28, 38]. In order to assess the ability of the generated Met-NTFs to bind its ligand, we took advantage of the first kringle domain (K1) of HGF/SF that we recently produced by total protein chemical synthesis [39]. Indeed, K1 is part of the high affinity HGF binding site for Met [40]. Biotinylation of K1 provided a probe that could be specifically precipitated with streptavidin beads (Figure 6A). K1 subdomain was able to capture the Met-NTFs both in MCF10A and in MDA-MB231 cells. The fragments were detected using antibodies directed either against α and β chain or *β chain* of the Met extracellular domain (Figure 6B). K1 subdomain was also able to capture recombinant Met extracellular region fused to Fc domain diluted in medium as control. Thus, when released in the extracellular medium, the Met-NTFs are able to recognize and efficiently bind HGF/SF subdomain, suggesting that they act as decoy molecules.

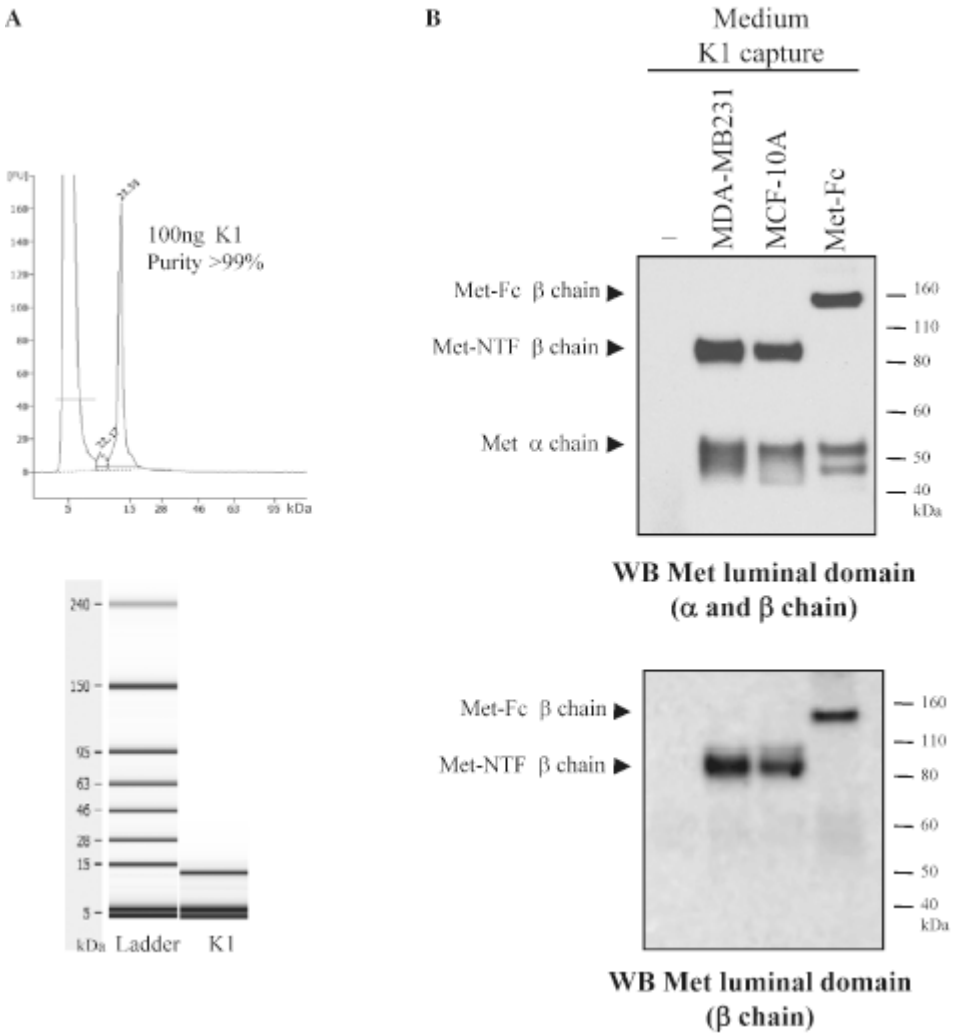

Figure 6. Association of the shedding-generated Met N-terminal fragment with K1 the high-affinity subdomain of HGF. A) High purity of the biotinylated-K1 produced by total chemical synthesis has been confirmed using the High Sensitivity lab-on-ship Bioanalyser Methods (purity>99%). Biotinylated-K1 electrophoregram and corresponding virtual gel with reference ladder are displayed. B) Serum depleted conditioned mediums of MCF-10A and MDA-MB231 and non-conditioned mediums containing or not recombinant Met extracellular region fused to Fc domain (Met-Fc) were collected and subjected to K1 capture. Proteins were resolved by 4–12% SDS-PAGE and analyzed by western blotting with antibodies directed against α and β chain or β chain of Met luminal domain.

DN30-mAb induced Met degradation through the lysosomal and γ-secretase pathways

The mAb DN30, directed against the extracellular region of human Met, is known to induce ectodomain shedding from the Met receptor[41]. In addition, we have demonstrated that Met downregulation by DN30 involves PS-RIP with γ-secretase cleavages and proteasomal degradation [23]. This inhibitory antibody thus hampers the ligand-independent biological activity triggered by overexpressed Met including neoplastic transformation and tumor growth.

Having demonstrated that Met-CTFs can be directly degraded by lysosomes, we evaluated the involvement of this degradation pathway in DN30-induced Met downregulation. Treatment of MCF-10A cells with the antagonistic DN30 antibody induced Met downregulation with a concomitant increase of Met-CTFs stabilized by either a lysosome or a γ-secretase inhibitor (Figure 7). Co-treatment with both inhibitors strongly increased the amount of Met-CTFs appearing in response to DN30 (Figure 7). Thus, the induction of Met shedding by this antibody promotes the generation of Met-CTFs, which are degraded both via the lysosomal pathway and through γ-secretase cleavage.

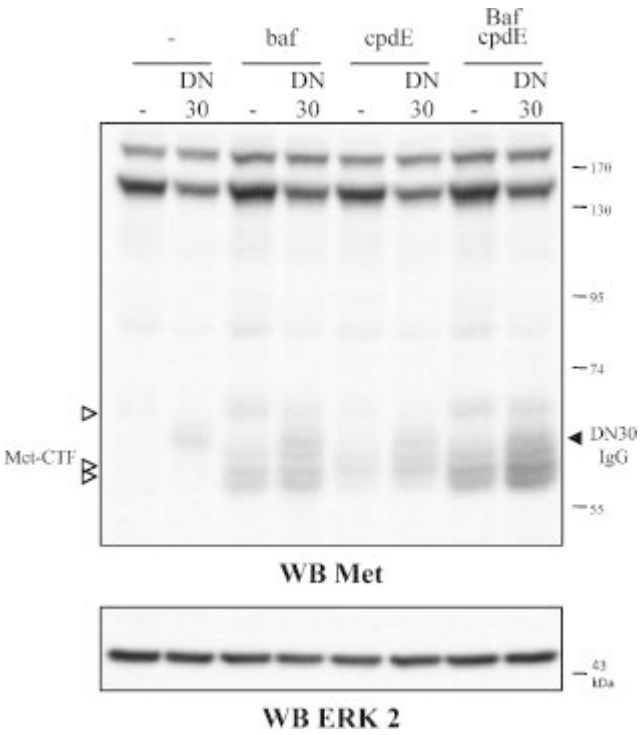

Figure 7. Induction of the lysosomal and the proteasomal degradation pathway by DN30 antibody. MCF-10A cells were treated for 16 h with 5 nMbafilomycin A1, 1 μM compound E, and/or 40 μg/mL DN30 antibody (DN30) before lysis. For each treatment, the same amount of proteins was resolved by 10% SDS-PAGE and analyzed by western blotting with antibodies directed against the kinase domain of Met and against ERK2. Arrows indicate the positions of the Met C-terminal fragments (Met-CTF) and of DN30 IgG.

Discussion

The degradation of RTKs is a crucial process downregulating their activity. The Met receptor, like other RTKs, can be degraded either in response to ligand activation or in a constitutive manner, independently of any stimulation [42].

We recently demonstrated that the Met receptor is degraded independently of ligand stimulation, through presenilin-dependent regulated intramembrane proteolysis. This proteolytic process is initiated by ectodomain shedding, i.e. the generation of Met-NTFs that are released into the extracellular medium and of C-terminal membrane-anchored fragments. These Met-CTFs are then processed by the γ-secretase complex, known to cleave receptors within the transmembrane domain. The generated intracellular domain of Met is, in turn, degraded by the proteasome. In contrast to HGF/SF-induced degradation, PS-RIP is constitutive and does not involve Met kinase activity. Uncleavable receptor displayed oversignaling, demonstrating that constitutive degradation of Met by PS-RIP prevents unregulated Met activation [23].

Ligand-induced degradation of Met is dependent to the receptor phosphorylation, leading to its ubiquitination, internalization and trafficking from clathrin-coated pit vesicles to late endosomal compartments. The full-length receptor is then degraded within the lysosome [42]. Defects in this process lead to oversignaling, indicating that ligand-dependent degradation of Met allows attenuation of the receptor responses [13]. Although the role of the lysosome in Met degradation is well established, Met degradation is also decreased by proteasomal inhibitors, to which other RTKs such as EGFR are insensitive. However, it has been shown that sensitivity of Met degradation to proteasome inhibitors is the consequence of promotion of recycling pathway from early endosomes at the expense of the sorting to late endosomes [43], suggesting that proteasome is not directly involved in degradation of ligand-activated full length Met. In contrast, the intracellular cytoplasmic domain (ICD), generated by γ-secretase cleavage in the PS-RIP process, is efficiently stabilized by proteasome inhibitors [23, 41], while lysosome inhibitors are ineffective, suggesting its direct degradation by proteasome

In this report, we demonstrate the existence of a second pathway involved in constitutive (ligand-independent) degradation of fragments generated through shedding: lysosomal degradation of both Met-NTFs and Met-CTFs. Whereas the Met shedding that initiates PS-RIP leads to release of the Met-NTF into the external medium, the Met-NTFs involved in the new pathway can be stabilized inside the cells by treatment with a lysosome inhibitor. This suggests that PS-RIP is initiated by Met shedding at the cell surface, but that the cleavage leading to lysosomal degradation affects intracellular Met. Accordingly, Met is found in the lysosomal compartment of lysosome-inhibitor-treated cells. In addition, biotin staining of the membrane proteins shows that the N-terminal region of Met degraded by the lysosome derives from a membrane-anchored receptor. Thus, membrane-bound full-length Met can be internalized and then cleaved within the juxtamembrane region. This intracellular shedding leads to generation of both Met-NTFs and Met-CTFs, which are in turn degraded in the lysosome. In keeping with this view, the Met-CTFs generated by intracellular shedding escape processing by the γ-secretase complex.

Taken together, these and previous data show that the cleavage associated with Met ectodomain shedding can initiate either direct degradation in the lysosome or classical PS-RIP leading to proteasomal degradation. We propose that routing to these two different degradation pathways depends on the subcellular localization of Met: when Met present at the cell surface sheds its ectodomain into the surrounding medium, this leads to γ-secretase cleavage of the membrane-anchored Met-CTF, but when Met present in intracellular vesicles is similarly cleaved, both resulting fragments undergo lysosomal degradation (Figure 8). The proteasome and the lysosome are involved in degradation of distinct fragments. Indeed co-treatment with proteasome and lysosome inhibitors leads to stabilization of distinct Met fragments (i.e Met-CTF and Met-ICD). In addition, this co-treatment did not induce synergistic stabilization of Met-CTF, indicating that proteasome is not or only weakly involved in the Met-CTF degradation. However, both degradation mechanisms participate in ligand-independent downregulation of Met. Indeed, inhibition of both lysosome and γ-secretase cleavage induces synergistic stabilization of Met-CTF, the common step of these degradation processes.

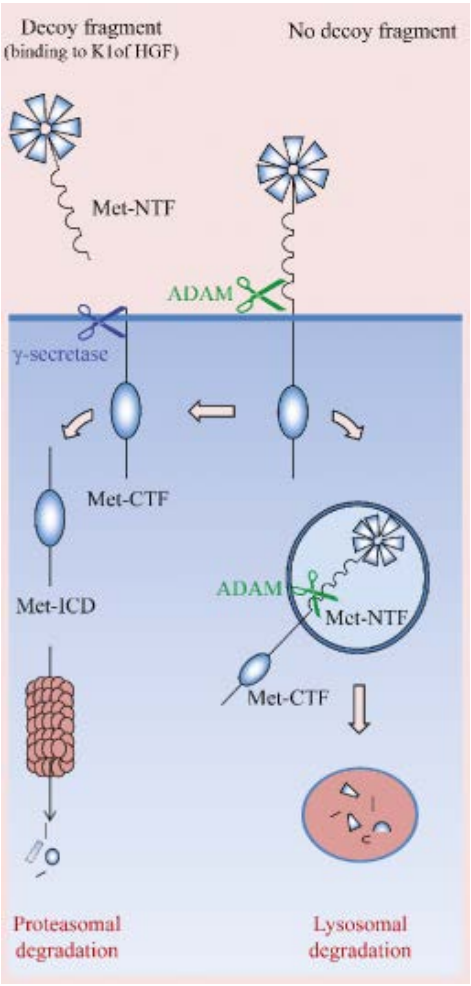

Figure 8. Schematic representation of ligand-independent Met degradation through PS-RIP or lysosome upon shedding. Independently of ligand stimulation, Met receptor can be degraded by the presenilin-regulated intramembrane proteolysis (PS-RIP). The action of ADAM-family membrane metalloproteases causes Met to shed an extracellular N-terminal fragment (Met-NTF), which can act as decoy fragment for HGF/SF. The membrane-anchored C-terminal counterpart (Met C-terminal fragment, Met-CTF) is then cleaved by the γ-secretase complex, generating a Met intracellular domain (Met-ICD), degraded by the proteasome. Upon shedding, both Met-NTF and Met-CTF can also be degraded within the lysosome, involving Met internalization and absence of further cleavage of Met-CTF by γ-secretase. In that case, lysosomal degradation of Met-NTF could occur at the expense of their release in the extracellular medium, which unfavors its potential decoy function.

The Met-CTFs stabilized by lysosome and γ-secretase inhibitors are contained between 55 and 60 kDa with three main detectable fragments. This smeary detection could be the consequence of post-traductional modifications including phosphorylation. However, Met-CTFs were not found tyrosine phosphorylated (data not shown). Alternatively, Met-CTFs can be generated by cleavages at several sites. Accordingly, Met shedding involves at least two metalloproteases, since silencing of ADAM10 [24, 25] and ADAM17 [23] inhibited partially the receptor cleavages. Interestingly, lysosome inhibitors stabilize Met-CTFs being shorter than upon γ-secretase inhibition. This suggests that intracellular and membrane shedding do not involve strictly similar cleavages by metalloproteases. Although we demonstrated that the first 50 amino acids of extracellular juxtamembrane of Met are necessary for the shedding, the cleavage sites remain to be characterized.

Several studies suggest that released Met N-terminal domains are decoy fragments. Indeed, the conditioned medium from various cell lines, releasing Met NTF, were able to inhibit HGF/SF stimulation evaluated indirectly through activation of signaling proteins downstream of Met such as AKT [28, 41]. Nevertheless, the property of the Met-NTFs to directly interact with HGF/SF is controversial [28, 38]. We demonstrate here that K1, a part of the high-affinity HGF binding site for Met, is able to capture in the medium the released Met-NTF. This suggests that when released in the external medium, Met-NTF is a decoy fragment. It is tempting to speculate that direct degradation of Met-NTF in lysosome could be a mechanism to reduce their extracellular release and decoy activity.

Although the development of inhibitory antibodies targeting the extracellular domains of membrane oncogenes is a promising therapeutic approach, the molecular mechanisms underlying the activity of such antibodies are not fully understood. We previously showed that the anti-Met antibody DN30, known to induce shedding of Met and downregulation of membrane-anchored Met, promotes its PS-RIP [23, 41]. We demonstrate here that DN30 antibody also induces direct lysosomal degradation of Met fragments. Thus, forced induction of Met shedding by DN30 actually targets Met to two complementary degradation pathways, which could explain the efficient degradation induced by this antagonistic antibody.

The lysosomal degradation of the Met-CTFs is original compared to the widely described proteasomal degradation of fragments from other membrane receptors. Nevertheless, this process does not seem to be restricted to the Met receptor. Indeed, during melanogenesis, the pigment cell-specific type I integral membrane protein (PMEL) is cleaved by a PS-RIP related process involving sequential cleavage by a site 2 protease and γ-secretase. Interestingly, these cleavages do not occur at the plasma membrane but in melanosome, a lysosomal related organelle. It has been recently demonstrated that PMEL C-terminal fragments are stabilized by inhibitors of lysosomal enzymes and accumulate in Lamp1 positive compartments upon γ-secretase inhibitors treatment, demonstrating their sorting to lysosome for degradation[44]. When type I membrane proteins are processed at plasma membrane, the C-terminal fragments are in turn cleaved by γ-secretase to create cytoplasmic intracellular fragments efficiently degraded by the proteasome. In contrast, in light of Met and PMEL processing, we can propose that when regulated intramembrane proteolysis occur in intracellular vesicle, the generated Met-CTF can be sorted to lysosome for further degradation. It is worth noticing that in contrast to Met receptor, the internalized C-terminal fragment of PMEL does not escape to γ-secretase cleavage, suggesting that in this case both PMEL-CTF and ICD might be degraded by lysosome.

Although at least 15 RTKs and several type I membrane proteins are cleaved in the extracellular juxtamembrane domain [29], direct lysosomal degradation of the generated C-terminal fragment has never been described. Yet although in most cases of receptor shedding, the fragment that remains is then processed by γ-secretase and degraded by the

proteasome, there are some receptors for which this second cleavage has not been reported. In such cases, direct lysosomal degradation of the generated fragments might be involved in their downregulation.

Materials and Methods

Cytokines, drugs and cell cultures

Human recombinant HGF/SF was purchased from Peprotech. The inhibitors of H+−pumps, bafilomycin A1 and concanamycin A, were purchased from Calbiochem. Chloroquine was purchased from Sigma–Aldrich. The proteasome inhibitor lactacystin was purchased from Sigma. The γ-secretase inhibitor compound E was purchased from Alexis/Coger. The γ-secretase inhibitors L-685,458 and DAPT, and the Met kinase inhibitor PHA665752 were purchased from Calbiochem. MDCK (Madin-Darby Canine Kidney Cells), HeLa, MCF-10A epithelial cells and NIH-3T3 fibroblast cells were cultured as previously described [23]. A549 cells, from epithelial lung tumor, were cultured in F-12K nutrient mixture medium (Invitrogen) with 10% fetal calf serum (FCS). MDA-MB-231 cells were cultured in DMEM, 10% FCS. MEF (murine embryonic fibroblasts) cells, WT or deficient for presenilin-1 and presenilin-2 were kindly provided by Paul Saftig (Christian-Albrechts-Universität, Germany) and Bart deStrooper (VIB, the Flanders Institute for Biotechnology, Belgium).

Antibodies

Mouse monoclonal antibody directed against C-terminal domain of mouse Met (B-2) and rabbit polyclonal antibody directed against C-terminal region of human Met (C-12) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody directed against kinase domain of Met (3D4) and C-terminal domain of human Met (L41G3) were, respectively, purchased from Invitrogen and Cell Signaling Technology. Rabbit antibody directed against phosphorylated tyrosine of the Met kinase domain was purchased from BioSource. Antibody directed against extracellular region of Met (DL-21) and DN30 antibody was previously described [45]. Antibody directed against ERK2 was purchased from Santa Cruz Biotechnology. Antibody against LAMP-1 (ab24170) was purchased from Abcam. Peroxidase and rhodamine conjugated antibodies directed against rabbit and mouse IgG were purchased from Jackson Immunoresearch Labs. Green-fluorescent Alexa fluor 488 conjugated anti-mouse IgG (H+L) and red-fluorescent Alexa fluor 594 conjugated anti-rabbit IgG (H+L) were purchased from Invitrogen. Biotin conjugated anti-rabbit IgG (H+L) was purchased from Jackson Immunoresearch Labs.

Plasmid constructions and transfection

The TRK-Met chimera expressing vectors were constructed as previously described [23]. Transient and stable transfections of MDCK cells were performed as previously described using the lipofection method [46].

Western blotting and immunofluorescence

Western blotting and immunofluorescence were performed as previously described [20]. For Western blot analysis of conditioned medium, MDA-MB231, HeLa (3.5×10^6 cells/100 mm-dish) and MCF10A (1×10^6 cells/100 mm-dish) cells were cultured in DMEM-10% FCS. The next day, cells were starved overnight in serum-free medium. Conditioned media were then collected (4 mL/100 mm plate) and the trypsinized cells were counted. The volumes of media were normalized to the cell numbers and centrifuged 20 min 3000× *g* at 4°C. Four volumes of cold acetone were added, incubated 1 h at −20°C and centrifuged 30 min 3000× *g* at 4°C. The pellet was suspended in 200 µL of RIPA buffer before western blot analysis.

For immunofluorescence, cells were incubated with a combination of a mouse antibody directed against kinase domain of Met (1:200) and a rabbit antibody against LAMP-1 (1:200). Cells were then incubated 60 min with a combination of green-fluorescent Alexa fluor 488 conjugated anti-mouse IgG (H+L) (6 mg/mL) (1:1000) and red-fluorescent Alexa fluor 594 conjugated anti-rabbit IgG (H+L) (6 mg/mL) (1:1000) (Invitrogen). Fluorescence was examined using confocal LSM 710 (Zeiss). Results are representative of at least three experiments.

Small interfering RNA

MCF-10A cells were cultured (2 x 10^5 cells/35 mm-dish) in DMEM-10% FCS. The next day, cells were incubated for 6 h in serum-free OptiMEM, 5 µL transfectant (Lipofectamine 2000; Invitrogen) and 50 nM small interfering RNA (siRNA; Invitrogen) targeting the Met (sequence 1: 5' AAAGAUAAACCUCUCAUAAUG 3' and sequence 2: 5" CAUUAUGAGAGGUUUAUCUUU 3') or a scrambled sequence. The cells were rinsed with DMEM-10% FCS before further treatment.

Cell-surface biotinylation

HeLa cells were cultured (2 x 10^6 cells/100 mm-dish) in DMEM-10% FCS. The next day, the cell-surface proteins were biotinylated with the Amersham ECL Protein biotinylation module (RPN 2202) according to manufacturer's instructions (GE Healthcare). Briefly, cells were incubated with 0.1 mg/mL biotinamidocaproate N-hydroxysuccinamide ester for 30 min on ice. The reaction was quenched by washing the cells with ice-cold PBS. The cultures were then stimulated by adding prewarmed medium (at 37°C) containing indicated inhibitors for 4 h before lysis.

Met-NTF affinity pool-down

MDA-MB231 (3.5 x 10^6 cells/100 mm-dish) and MCF10A (1 x 10^6 cells/100 mm-dish) cells were cultured in DMEM-10% FCS. The next day, cells were starved overnight in serum-free medium. Conditioned media were then collected and the trypsinized cells were counted. The volumes of media were normalized to the cell numbers and concentrated into Vivaspin 2 cut off 10 kDa ultrafiltration columns (Sartorius) up to a final volume of ~800–1000 µL. Streptavidine-Sepharose beads (GE Healthcare) were washed and equilibrated in PBS. Beads were loaded with 50 µg of biotinylated K1 (200 µL beads in a 50:50 PBS:beads slurry) for 20 min at room temperature and immediately wash with PBS. Beads were incubated in conditioned culture media overnight at 4°C under mild agitation (25 µL beads per assay). Beads were quickly washed with PBS and bound proteins were eluted with 200 mM Glycine buffer pH = 2. Elution fractions were next analyzed by SDS-PAGE and subsequent western blotting.

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