The Met Receptor Degradation Pathway

REQUIREMENT FOR LYS48-LINKED POLYUBIQUITIN INDEPENDENT OF PROTEASOME ACTIVITY*

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Acute stimulation of the receptor for the hepatocyte growth factor/scatter factor Met leads to receptor ubiquitination and down-regulation through the lysosomal degradation pathway. We have determined that the Met receptor undergoes multiple monoubiquitination as opposed to the appendage of polyubiquitin chains. Nevertheless, overexpression of ubiquitin in HEK293T cells enhances the rate of Met receptor degradation, in contrast to a point mutant of ubiquitin (K48R) that cannot form Lys48-linked polyubiquitin chains. Furthermore, an enhancement of Met degradation is also seen under conditions where the proteasome is inhibited by lactacystin. We propose that this reflects polyubiquitin-dependent sorting of Met, as the overexpression of ubiquitin but not K48R ubiquitin also restores hepatocyte growth factor-dependent phosphorylation of the endosomal coat protein Hrs from inhibition by lactacystin. Our data indicate a requirement for K48-R-linked polyubiquitin for Met endosomal trafficking independent of its canonical function of targeting for proteasomal degradation.

The receptor for hepatocyte growth factor (HGF)1/scatter factor (SF), c-Met, is a tyrosine kinase that, upon stimulation, elicits a variety of cellular responses. Collectively, these responses may be thought of as components of a program of “invasive growth” that are expressed according to cellular context and the developmental stage of the organism (1). Aberrant c-Met signaling is likely to contribute toward tumor progression and metastasis. Elevated c-Met expression is found in many late stage tumors and is often an indicator of a poor prognosis (2). A variety of oncogenic germ line mutations have been isolated from sporadic tumors such as renal papillomas and gastric carcinoma (3–5).

Previous studies have shown that acute HGF stimulation leads to down-regulation of the receptor (6). This degradation pathway accompanies receptor endocytosis and is sensitive to the proton pump inhibitor bafilomycin (7), which indirectly inhibits acid-dependent lysosomal proteases. The HGF-dependent degradation pathway is also sensitive to inhibitors of the 26 S proteasome such as lactacystin, which appear to exert their effect through the perturbation of c-Met trafficking to late endosomal degradative compartments (7–9). In this respect the degradation pathway of Met differs from several other tyrosine kinase receptors such as the platelet-derived growth factor receptor and the epidermal growth factor receptor (EGFR), which are relatively insensitive to lactacystin (10, 11). Interestingly, in the case of EGFR, lactacystin inhibits the transfer of EGFR to luminal vesicles of multivesicular bodies while still allowing sorting to the limiting membrane of late endosomes/lysosomes, where the receptor can be degraded (11).

For many receptors, ubiquitination plays a key role in their endosomal trafficking. Several yeast receptors and transporters (e.g. the α-factor receptor (Ste2p) and the uracil permease (Fur4p)) require ubiquitination as a targeting signal for endocytosis and subsequent degradation (reviewed in Ref. 12). Indeed, in yeast all endogenous proteins, which are known to be internalized, use ubiquitin as an internalization signal. For certain receptors (e.g. the α-factor receptor), monoubiquitination alone is sufficient for signal internalization (13). Such a modification is distinct from that required for targeting to and degradation by proteasomes, where polyubiquitination resulting in a chain of at least four ubiquitin molecules is required (14). Ubiquitin can form chains expressing different functions by linking through different lysines. To specify proteasomal degradation a Lys48-linked polyubiquitin is required, whereas Lys63-linked chains participate in the coordination of error-free DNA repair (15).

Activation of tyrosine kinase receptors such as EGFR and Met results in ubiquitination that manifests itself as a higher molecular weight smeared band suggestive of the addition of several ubiquitin moieties. In the case of EGFR and platelet-derived growth factor receptor, this effect has been shown to be due to multi-monoubiquitination rather than an extension of polyubiquitin chains, but to date this finding has not been confirmed for other tyrosine kinase receptors (16, 17). Ubiquitination of Met and EGFR are both dependent on the E3 ubiquitin ligase Cbl (18, 19), yet their degradation pathways show differential sensitivity to lactacystin. In this paper we demonstrate that, in common with the EGFR, the Met receptor is multi-monoubiquitinated. Nevertheless, in contrast to EGFR, Met degradation appears to require the cellular capacity for the production of Lys48-linked polyubiquitin chains. Remarkably, we find that Lys48-linked ubiquitin enhances Met degradation under conditions where proteasome activity is blocked, suggesting a novel function for this type of linkage.

MATERIALS AND METHODS

Cell Culture—All cell culture reagents were obtained from Invitrogen. HeLa and HEK293T cells were incubated in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 1% non-essential amino acids, and 100 units/ml penicillin and streptomycin sulfate with 5% CO2 at 37 °C.

Antibodies and Other Reagents—Purified recombinant human HGF/SF was a kind gift from Dr. George Vande Woude (Van Andel...
Lactacystin and concanamycin were obtained from Merck Biosciences. The antibodies used were the anti-Met Hu intracellular domain antibody (25H2) from Cell Signaling Technology, the anti-Met Hu extracellular domain antibody (DO-24) from Upstate Biotechnology Inc., mouse monoclonal anti-\(\beta\)-catenin and anti-phospho-tyrosine (PY20) antibodies from BD Transduction Laboratories, the anti-tubulin antibody from Sigma, and the anti-RGS-His antibody from Qiagen. Anti-ubiquitin antibodies were obtained from Sigma (rabbit polyclonal antibody; U-5379), Covance (mouse monoclonal antibody; P4G7), and Affiniti (FK1 and FK2 monoclonal antibodies). The rabbit polyclonal anti-Hrs antibody has been described previously (9).

**Molecular Biology and Transfections**—Wild-type and K48R His\(_6\)-ubiquitin-GFP constructs were generous gifts from Doug Gray (Ottawa Regional Cancer Centre, Ottawa, Canada). A K29R/K48R/K63R and K63R mutant were created by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). HEK293T cells were transfected using GeneJuice (Novagen) for 39 h.

**Ubiquitin Detection**—To resolve free monomeric endogenous and exogenous ubiquitin, a modified SDS-PAGE/Western blotting protocol was used as described previously (20). Total ubiquitinated proteins were analyzed by resolving lysate proteins on 5 or 8% SDS-PAGE gels followed by transfer to nitrocellulose membranes and probing with either an anti-ubiquitin antibody (P4G7) or an anti-RGS-His antibody.

**\(\beta\)-Catenin Degradation**—Following transfection, cells were incubated either with or without 10 \(\mu\)M lactacystin for 6 h. Cells were then lysed, resolved by SDS-PAGE, and transferred to nitrocellulose membranes for Western blot analysis.

**Met Down-regulation, Ubiquitination, and HGF-dependent Hrs Phosphorylation**—Met down-regulation was measured as described previously for HeLa cells using anti-Met \(^{\text{Intracellular}}\) (25H2) for detection (7). Met ubiquitination and Hrs phosphorylation were determined as described previously (9). For ubiquitin experiments, lysis buffer was also supplemented with 10 mM \(N\)-ethylmaleimide where required.

**RESULTS AND DISCUSSION**

### HGF-dependent Multiple Monoubiquitination of the Met Receptor

The anti-ubiquitin monoclonal antibodies FK1 and FK2 differ in their ability to recognize polyubiquitinated proteins. FK1 only recognizes polyubiquitinated proteins, whereas FK2 recognizes both polyubiquitin and monoubiquitin appended to proteins (21). Thus, these reagents can be used to discriminate monoubiquitination from polyubiquitination, as exemplified by studies that established EGFR monoubiquitination (16). Blotting cell lysates with either FK1 or FK2 shows similar band patterns, indicating that most ubiquitinated proteins are polyubiquitinated (Fig. 1A). Note that under the antibody dilutions presented, the intensity of the bands is higher with FK1. Using these same dilutions we probed anti-Met immunoprecipitates derived from HeLa cell lysates. No bands could be detected with FK1, but an HGF-dependent ubiquitinated Met smear was clearly detected with FK2. The smeary appearance of this signal indicates multi-monoubiquitination of the Met receptor following HGF stimulation (Fig. 1B).

### K48R-linked Polyubiquitin Enhances Met Degradation

We have previously demonstrated a role for the 26 S proteasome in...
sorting the Met receptor to the lysosomal pathway (7, 9). Therefore, a requirement for polyubiquitination and the consequent proteasomal degradation of an accessory factor cannot be discounted. In principle, cellular events requiring polyubiquitin chain formation can be inhibited by the overexpression of forms of ubiquitin-bearing lysine mutations that terminate chain assembly. We have used ubiquitin that contains either single K48R or K63R mutations or ubiquitin containing three mutations (K29R/K48R/K63R, referred to as mono-Ub). This latter mutant lacks any lysine residues known to have a role in polyubiquitin chain formation in vivo. In practice, this approach can suffer from the high levels of endogenous ubiquitin expression, which has been estimated to be up to 20 μM (22). A further complication is the abundance of cellular de-ubiquitinating enzymes, which may render ubiquitin chains unstable. Nevertheless, the expression of K63R Ub has been shown to sensitize cells to protein-damaging agents (23).

We have transiently transfected HEK293T cells with His₆-ubiquitin-GFP constructs (23). Following synthesis, the GFP portion of this chimeric protein is cleaved to yield free His₆-ubiquitin at levels approximately equal to those of endogenous ubiquitin (Fig. 2A, bottom). The His-tagged form is slightly larger than the endogenous ubiquitin and, therefore, can be resolved from endogenous ubiquitin on a 15% gel. Wild-type, K48R, and mono-Ub plasmids express approximately equal levels of free His-ubiquitin, and all three appear to be incorporated into proteins with similar efficiency (Fig. 2A, top). Overexpression of wild-type or mutant forms of ubiquitin also leads to a moderate increase in total ubiquitinated proteins (Fig. 2A, middle). Crucially, HEK293T cells can be transfected with close to 100% efficiency, allowing clear analysis of the effects of mutant ubiquitin on endogenous receptors without recourse to co-expression, which introduces a number of technical difficulties. In these cells the HGF-dependent degradation of the Met receptor displays sensitivity to the proteasome inhibitor lactacystin and the lysosomal protease inhibitor concanamycin (Fig. 2B), albeit not as profoundly as we have previously observed in HeLa cells (7).

To demonstrate the biological effects of mutant ubiquitin expression we looked at levels of β-catenin, a known substrate of the ubiquitin–proteasome pathway (24) (Fig. 2C). We can detect clear differences in the pattern of lower molecular mass bands both in the absence and the presence of lactacystin. Most notably, K48R-Ub completely inhibits the generation of a 50-kDa band that is accumulated upon lactacystin treatment. In untreated cells, levels of an 80-kDa band are decreased by the expression of Ub and increased by the expression of K48R-Ub. This band may be an unmodified form, which is accumulated in cells where the proteasomal turnover of polyubiquitinated proteins is compromised.

In HEK293T cells the degradation rate of endogenous Met is significantly increased by the expression of wild-type or K63R-Ub but not mono-Ub, suggesting that polyubiquitin chain formation stimulates Met degradation (Fig. 3A–C). This observation is in direct contrast to the case of EGFR, where similar experiments showed that mono-Ub was equipotent with the wild-type in enhancing EGFR degradation (16). K48R-ubiquitin also failed to fully recapitulate the enhancement of Met receptor degradation provided by the overexpression of wild-type ubiquitin. After 2 h of acute stimulation with HGF, the percentage amount of Met receptor remaining for mock transfected cells is 23.75% (± 2.1%), which is reduced to 6% (± 1.2%) when wild-type ubiquitin is overexpressed. The corresponding values following overexpression of mono-Ub and K48R-Ub are 27.25 (± 4.8%) and 13.75% (± 0.5%) respectively (n = 4). No reduction in the degree of Met ubiquitination was observed in cells transfected K48R or mono-Ub compared with wild-type (Fig. 3D).

**FIG. 2. Expression of His₆-ubiquitin-GFP constructs in HEK293T cells.** A. HEK293T cells were mock transfected or transfected with wild-type, K48R or mono-Ub. Cells lysates were analyzed by immunoblotting (IB) with anti-RGS-His (i), anti-ubiquitin (P4G7) (ii), or anti-ubiquitin (U-5379) (iii). B. HEK293T cells were pre-treated with lactacystin or concanamycin and then cultured in medium with (+) or without (–) HGF/SF for the required times. Cell lysates were analyzed by immunoblotting (IB) with anti-Met (25H2). C. HEK293T cells were mock transfected or transfected with either wild-type or K48R Ub. Cells were then cultured in medium with or without lactacystin for 6 h. Cell lysates were analyzed by immunoblotting (IB) with anti-β-catenin.

Lys₄₈-linked Ubiquitin Enhances Met Receptor Degradation under Conditions of Proteasome Inhibition—We have also studied the effect of the overexpression of wild-type and K48R-Ub on Met down-regulation in cells that have been pre-treated with the proteasome inhibitor lactacystin (Fig. 4A). The expression of wild-type Ub reduces the amount of Met remaining after 4 h of acute stimulation in the presence of lactacystin by ~6-fold, whereas K48R-Ub has a markedly weaker effect (~2-fold). Surprisingly, this result indicates a potential role in Met degradation for Lys₄₈-linked polyubiquitin in the absence of proteasome activity.

We have shown previously that the majority of Met receptors are degraded in the lysosome. In HeLa cells more than two-thirds of the receptors follow this pathway to degradation, but...
the presence of other degradation pathways is evident. In the case where endocytosis is blocked by the expression of mutant dynamin, degradation of the receptor still occurs at a slower rate and can be partially blocked by lactacystin (7). It is possible that Ub expression is enhancing this pathway rather than the endosomal route. One signature of the correct endosomal sorting of activated Met is the phosphorylation of the endosomal protein Hrs, which may reflect capture in a clathrin-coated region of the sorting endosome. Lactacystin treatment inhibits HGF- but not EGF-dependent Hrs phosphorylation (9). We have now used this assay to show that wild-type but not K48R-Ub can restore HGF-dependent Hrs phosphorylation in the presence of lactacystin (Fig. 4B).

**FIG. 4. Proteasomal activity is not essential for Met down-regulation.** A, HEK293T cells were mock transfected or transfected with wild-type or K48R Ub and then cultured in medium with (+) or without (−) HGF/SF for 4 h. Where indicated, cells were pre-incubated with lactacystin. Cells lysates were analyzed by immunoblotting (IB) with anti-Met (25H2) and anti-tubulin. B, HEK293T cells were mock transfected or transfected with wild-type, K48R, or mono-Ub and then cultured in medium with (+) or without (−) HGF/SF for 8 min. Where indicated, cells were pre-incubated with lactacystin. Cells were lysed, and Hrs was immunoprecipitated (IP) and analyzed by immunoblotting (IB) with PY20 or anti-Hrs antibodies.

**DISCUSSION**

Our data indicate that, similar to other tyrosine kinase receptors (EGFR and the platelet-derived growth factor receptor), stimulation of the Met receptor leads to multiple monoubiquitination rather than the attachment of polyubiquitin chains to the receptor. Multi-monoubiquitination of receptors such as EGFR and Met is likely to be required for the sorting of these receptors at the early endosome by interaction with ubiquitin interaction motif-containing proteins that are components of the sorting machinery such as Hrs, STAM, eps15, and epsin (25). In fact, the appendage of a single non-cleavable form of ubiquitin to the EGFR is sufficient to ensure efficient lysosomal targeting (16).

A pathway utilizing monoubiquitin as a sorting signal will not require the active involvement of the proteasome other than to maintain free ubiquitin levels. We have previously found that the degree of HGF-dependent Met receptor ubiquitination is not significantly changed by proteasome inhibition (9). Nevertheless, Met receptor degradation is exceptionally sensitive to proteasomal inhibition, in comparison with the other tyrosine kinase receptors that have been characterized to date. However, studies of non-tyrosine kinase receptors such as the growth hormone receptor and the interleukin 2 receptor have also shown a requirement of proteasomal activity for endocytosis and/or subsequent sorting into the lysosomal pathway (26, 27). Interestingly, ubiquitination of the growth hormone receptor itself appears to be dispensable for down-regulation, which has led to a model in which polyubiquitination

**FIG. 3. Met down-regulation requires the formation of Lys^48- linked polyubiquitin chains.** A, HEK293T cells were transfected with wild-type or mono-Ub. Cells were then cultured in medium with or without HGF/SF for the required times. Cell lysates were analyzed by immunoblotting (IB) with anti-Met (25H2). B, intensity of the bands shown in panel A was quantitated using NIH Image 1.63 software. For each time point the percentage of Met remaining was calculated. Each column represents the mean ± S.E. of seven (1-h time point) or five experiments (2-h time point). C, HEK293T cells were transfected with wild-type, K48R, or K63R Ub. Cells were then cultured in medium with or without HGF/SF for the required times. Cell lysates were analyzed by immunoblotting with anti-Met (25H2). D, HEK293T cells were mock transfected or transfected with wild-type, K48R, or mono-Ub and then cultured in medium with (+) or without (−) HGF/SF for 5 min. Met was immunoprecipitated from cell lysates with anti-Met (DO-24) and analyzed by immunoblotting (IB) with anti-ubiquitin (P4G7) or anti-Met (25H2).
and proteasomal degradation of an unidentified accessory factor is required. α-Amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA)-type glutamate receptors also require ubiquitination and proteasomal degradation of the scaffolding protein PSD-95 in order to undergo endocytosis (28). Our previous findings that Met degradation requires both correct endosomal sorting and proteasome activity have also been proposed to reflect a requirement for degradation of an accessory factor (6).

Our data showing enhancement of Met receptor degradation by the expression of Ub but not mono-Ub represent a further departure from the EGFR degradation pathway (16). We must consider that this finding could be due to the promotion of other poorly defined endocytosis-independent degradation routes, which we have previously reported to be minor components of HGF-dependent Met degradation in HeLa cells (7). However, we show that the expression of Ub but not K48R-Ub can restore a lactacystin-imposed block to HGF-dependent phosphorylation of Hrs, which we have defined previously as a signature of the correct endosomal sorting of the Met receptor (9, 29). For this reason we prefer a model in which Lys48-linked polyubiquitin promotes lysosomal sorting of the Met receptor. This mechanism could correspond to the enhanced proteasomal degradation of the proposed accessory factor; but how then can we account for our observation that we retain Lys48-linkage-dependent enhancement of Met degradation under conditions where we have blocked proteasome activity with lactacystin? Does this represent an entirely novel function for Lys48-linked ubiquitin, which has previously been uniquely associated with a proteasomal targeting signal?

One scenario is that the critical step is the sequestration of a polyubiquitinated accessory factor away from Met, which is followed by incidental proteasomal degradation. In this model, proteasome activity is only required to generate ubiquitin (possibly locally), which can contribute to Lys48 chain formation. It has recently been suggested that polyubiquitin chains are assembled in the cytosol, added en bloc to ubiquitin interaction motif domain proteins, and then trimmed to generate monoubiquitinated proteins (31). The formation of Lys48-linked chains could thus promote the monoubiquitination of endosomal sorting factors, which, in turn, may influence Met receptor sorting.

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REFERENCES
1. Comoglio, P. M., and Boccaccio, C. (2001) Semin. Cancer Biol. 11, 153–165
2. Haddad, R., Lipsen, K. E., and Webb, C. P. (2001) Anticancer Res. 21, 4243–4252
3. Lee, J.-H., Han, S.-U., Cho, H., Jenninges, B., Gerrard, B., Dean, M., Schmidt, L., Zbar, B., and Vande Woude, G. F. (2006) Oncogene 19, 4847–4853
4. Jeffers, M., Schmidt, L., Nakaiwaga, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11445–11450
5. Oliviero, M., Valente, G., Bardelli, A., Longati, P., Ferrero, N., Cracco, C., Terrone, C., Rocca-Rossetti, S., Comoglio, P. M., and Di Renzo, M. F. (1999) Int. J. Cancer 82, 640–643
6. Hammond, D. E., Carter, S., and Clague, M. J. (2004) Curr. Top. Immunol. Microbiol. 286, 21–44
7. Hammond, D. E., Urbe, S., Vande Woude, G. F., and Clague, M. J. (2001) Oncogene 20, 2761–2770
8. Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S., and Vande Woude, G. F. (1997) Mol. Cell. Biol. 17, 799–808
9. Hammond, D. E., Carter, S., McCullough, J., Urbe, S., Vande Woude, G., and Clague, M. J. (2003) Mol. Biol. Cell 14, 1346–1354
10. Mori, S., Tanaka, K., Omura, S., and Saito, Y. (1995) J. Biol. Chem. 270, 29447–29452
11. Longva, K. E., Blystad, F. D., Stang, E., Larsen, A., M, Johannessen, L. E., and Madsen, J. H. (2002) J. Cell Biol. 156, 843–854
12. Hicke, L., and Dunn, R. (2003) Annu. Rev. Cell Dev. Biol. 19, 141–172
13. Shih, S. C., Sloper-Mould, K. E., and Hicke, L. (2000) EMBO J. 19, 187–198
14. Hershko, A., and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479
15. Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G., and Jentsch, S. (2002) Nature 419, 135–141
16. Haglund, K., Zsigmond, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dicke, I. (2003) Nat. Cell Biol. 5, 461–466
17. Mosehagen, Y., Shitiegm, K., Katz, M., Zhang, Y., Vereb, G., Szilosti, L., and Yarden, Y. (2003) J. Biol. Chem. 278, 21323–21326
18. Levkovitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) Genes Dev. 12, 3663–3674
19. Peschard, F., Fournier, T. M., Lamorte, L., Naojokas, M. A., Band, H., Langdon, W. Y., and Park, M. (2001) Mol. Cell 8, 995–1004
20. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
21. Fujimuro, M., Sawada, H., and Yokosawa, H. (1994) FEBS Lett. 349, 173–180
22. Haas, A. L., and Bright, P. M. (1987) J. Biol. Chem. 262, 345–351
23. Tsigitis, M., Zhang, M., Chiu, R. K., Wouters, B. G., and Gray, D. A. (2001) J. Biol. Chem. 276, 46973–46978
24. Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997) EMBO J. 16, 3797–3804
25. Polo, S., Confolanier, S., Salcini, A. E., and Di Fiore, P. P. (2003) Science’s STKE http://stke.sciencemag.org/cgi/content/abstract/sigtrans;2003/213/rev17
26. Strous, G. J., and van Kerkhof, P. (2002) Mol. Cell. Endocrinol. 197, 143–151
27. Rocca, A., Lamaze, C., Subtil, A., and Dautry-Varsat, A. (2001) Mol. Biol. Cell 12, 1293–1301
28. Colledge, M., Snyder, E. M., Crozier, R. A., Soderling, J. A., Jin, Y., Langerberg, L. K., Lu, H., Bear, M. F., and Scott, J. D. (2003) Neuron 40, 595–607
29. Urbe, S., Mills, I. G., Stenmark, H., Kitamura, N., and Clague, M. J. (2000) Mol. Biol. Cell 20, 7685–7692
30. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2003) J. Cell Biol. 162, 71–84
31. Miller, S. L., Malotky, E., and O’Bryan, J. P. (2004) J. Biol. Chem. 279, 33528–33537