Endosomal Dynamics of Met Determine Signaling Output

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Proteasomal activity is required for Met receptor degradation after acute stimulation with hepatocyte growth factor (HGF). Inhibition of proteasomal activity with lactacystin leads to a block in the endocytic trafficking of Met such that the receptor fails to reach late endosomes/lysosomes, where degradation by acid-dependent proteases takes place (Hammond *et al.*, 2001). In this article, we have biochemically determined Met internalization rates from the cell surface and shown that lactacystin does not inhibit the initial HGF-dependent internalization step of Met. Instead, it promotes the recycling pathway from early endosomes at the expense of sorting to late endosomes, thereby ensuring rapid return of internalized Met to the cell surface. We have used this perturbation of Met endosomal sorting by lactacystin to examine the consequences for HGF-dependent signaling outputs. In control cells HGF-dependent receptor autophosphorylation reaches a maximal level over 5–10 min but then attenuates over the ensuing 50 min. Furthermore, Met dephosphorylation can be kinetically dissociated from Met degradation. In lactacystin-treated cells, we observe a failure of Met dephosphorylation as well as Met degradation. Elements of the mitogen-activated protein kinase cascade, downstream of receptor activation, show a normal kinetic profile of phosphorylation, indicating that the mitogen-activated protein kinase pathway can attenuate in the face of sustained receptor activation. The HGF-dependent phosphorylation of a receptor substrate that is localized to clathrin-coated regions of sorting endosomes, Hrs, is dramatically reduced by lactacystin treatment. Reduction of cellular Hrs levels by short interfering RNA modestly retards Met degradation and markedly prevents the attenuation of Met phosphorylation. HGF-dependent Hrs phosphorylation and Met dephosphorylation may provide signatures for retention of the receptor in coated regions of the endosome implicated in sorting to lysosomes.

INTRODUCTION

The receptor for hepatocyte growth factor (HGF)/scatter factor (SF), Met, is a tyrosine kinase, which upon stimulation elicits a variety of cellular responses. These include mitogenesis, increased cell motility, and morphogenesis (e.g., tubule formation of kidney epithelial cells). Collectively, these may be thought of as components of a program of “invasive growth” that are expressed according to cellular context and developmental stage of the organism (Comoglio and Boccaccio, 2001). Aberrant Met signaling is likely to contribute toward tumor progression and metastasis. A variety of on-

cogenic germ line mutations have been isolated from sporadic tumors such as renal papillomas and gastric carcinoma (Jeffers *et al.*, 1997a). Elevated Met expression is found in many late stage tumors and is often an indicator of a poor prognosis (Haddad *et al.*, 2001).

Previous studies have shown that acute HGF stimulation leads to down-regulation of the receptor (Jeffers *et al.*, 1997b; Hammond *et al.*, 2001). This degradation pathway follows receptor endocytosis and is sensitive to the proton pump inhibitor concanamycin (Hammond *et al.*, 2001) that indirectly inhibits acid-dependent lysosomal proteases (Bowman *et al.*, 1988; Yoshimori *et al.*, 1991). The HGF-dependent degradation pathway is also sensitive to inhibitors of the 26S proteasome such as lactacystin (Jeffers *et al.*, 1997b; Hammond *et al.*, 2001), which seem to exert their effect through perturbation of Met trafficking to late endosomal degradative compartments (Hammond *et al.*, 2001). Normally, after 1-h stimulation of HeLa cells with HGF, immunofluores-

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cence microscopy reveals that the remaining Met receptor is associated with perinuclear punctae that presumably constitute a class of late endosomes. However, if cells are pretreated with lactacystin then the receptor seems confined to the plasma membrane (Hammond *et al.*, 2001). These observations do not formally distinguish between a block to receptor internalization and a block in transport to late endosomal compartments. This is because at steady state, a plasma membrane staining will be evident if receptor internalization is inhibited but if endosomal recycling is rapid compared with the internalization rate.

For other receptors lactacystin has been reported to variably block at the internalization step and/or at the endosomal sorting step. Internalization of growth hormone receptor (GHR) is blocked by lactacystin (van Kerkhof *et al.*, 2000), whereas another cytokine receptor, interleukin-2, is able to internalize but is then defective in sorting to late endosomes (Rocca *et al.*, 2001). Among tyrosine kinase receptors Met degradation seems to be especially sensitive to lactacystin treatment (Bonifacino and Weissman, 1998), but it has recently been shown that sequestration of epidermal growth factor (EGF) receptor into luminal vesicles of endosomal multivesicular bodies is proteasome activity dependent (Longva *et al.*, 2002). Met is itself ubiquitinated after HGF administration and, interestingly, an oncogenic form of Met has been shown to lack a binding site for the E3 ubiquitin ligase c-Cbl (Peschard *et al.*, 2001).

Ubiquitination of membrane proteins has been defined as a sorting signal for inclusion into luminal vesicles of multivesicular bodies and a number of proteins that contribute to the sorting process have been shown to bind ubiquitin (Katzmann *et al.*, 2001; Lloyd *et al.*, 2002; Polo *et al.*, 2002). One of these proteins, Hrs, has been shown to be significantly enriched (20 $\times$) at an area of the surface of the sorting endosome that is covered by a distinctive "bilayered" clathrin coat that also provides a site of concentration of receptors destined for late endosomes (Clague, 2002; Sachse *et al.*, 2002).

Localization to specific cellular compartments may present an enzyme with a distinct set of substrates unique to that compartment and hence receptor dynamics can determine signaling outcomes (Di Fiore and Gill, 1999; Clague and Urbé, 2001; McPherson *et al.*, 2001). This has been clearly shown, in the case of the EGF receptor, by blocking internalization of the receptor by expression of a dominant negative form of dynamin, which leads to differential effects on various signaling pathways downstream of receptor activation (Vieria *et al.*, 1996). Similarly, lactacystin, which blocks internalization of GHR, modifies the signaling of the activated receptor (Alves dos Santos *et al.*, 2001).

In this study, we have developed biochemical assays for internalization and recycling of Met. The internalization assay allows us to study early time points after stimulation. It reveals that lactacystin does not block internalization of Met but rather exerts its effect at the level of endosomal sorting. We have then analyzed the sensitivity of Met signaling outputs to lactacystin treatment. Our results show that correct endosomal sorting is required for the dephosphorylation of receptor that occurs after an HGF-dependent phosphorylation and for phosphorylation of Hrs, whereas the phosphorylation profile of the mitogen-activated protein (MAP) kinase pathway is insensitive to lactacystin.

MATERIALS AND METHODS

Cell Culture

All cell culture reagents were obtained from Invitrogen (Carlsbad, CA). HeLa cells were incubated in a humidified 5% CO₂ atmosphere, at 37°C, in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, and 100 U/ml both penicillin and streptomycin sulfate. K44A cells (Damke *et al.*, 1995) (generous gift of S. Schmid, Scripps Research Institute, La Jolla, CA) were cultured in HeLa medium supplemented with G418 (400 μ g/ml), puromycin (200 ng/ml), and tetracycline (1 μ g/ml). For induction of hemagglutinin (HA)-tagged K44A mutant dynamin, tetracycline was withdrawn for 48 h before the experiment. Expression of mutant dynamin was monitored by both Western blotting and immunofluorescence. Typically, $\geq 95\%$ of cells expressed the K44A mutant and the corresponding cells showed inhibition of biotinylated transferrin uptake as judged by labeling with streptavidin-Oregon Green 488.

Antibodies and Other Reagents

Two forms of purified recombinant human HGF/SF were used in this study. That used in cell signaling assays was purified from the supernatant of NIH3T3 cells engineered to overexpress the factor as described previously (Rong *et al.*, 1993). It consists of a mixture of predominantly single-chain inactive HGF/SF, as well as some heterodimeric HGF/SF. The extracellular serine protease required to cleave the single-chain propeptide forming the biologically active molecule has previously been shown to be produced either by the cells in culture or is present in serum (Naldini *et al.*, 1992). For biotinylated Met internalization assays a pure preparation of active heterodimeric HGF/SF was used and was obtained from Genentech (South San Francisco, CA). Purified human EGF was obtained from J. Smith (University of Liverpool, Liverpool, United Kingdom).

Lactacystin and clasto-lactacystin β -lactone were obtained from Calbiochem (San Diego, CA). Anti-Met^{Hu} intracellular domain antibody, a rabbit polyclonal antibody (sc-10), was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-Met^{Hu} extracellular domain antibody was obtained from Upstate Biotechnology (Lake Placid, NY). The rabbit polyclonal anti-Hrs antibody was raised against a C-terminal peptide of human Hrs (PPAQGSE⁴QLISFD). Its specificity was confirmed in competition experiments by immunoblotting of cell extracts and immunofluorescence of cells transiently overexpressing green fluorescent protein- and HA-tagged Hrs. Mouse monoclonal antibody against both mono- and polyubiquitinated protein conjugates (FK2) was obtained from Affinity Bioreagents (Boulder, CO). The anti-phosphotyrosine monoclonal antibody (PY20) was obtained from Transduction Laboratories (Lexington, KY). Monoclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (E10) and rabbit polyclonal anti-phospho-MEK1/2 (Ser217/221) and anti-phosphoMet (1349) antibodies were obtained from Cell Signaling Technology (Beverly, MA).

HGF/SF Stimulation and Downstream Signaling Assay

Cells were grown to $\sim 60\%$ confluence on 60-mm-diameter tissue culture dishes and stimulated for various time periods with ~ 250 ng/ml HGF/SF. Where indicated, cells were preincubated for 2 h with 10 μ M lactacystin, which also remained throughout the period of HGF/SF stimulation. After stimulation, the cells were washed several times with cold phosphate-buffered saline (PBS) and lysed for 10–15 min on ice in lysis buffer (0.5% NP-40, 25 mM Tris pH 7.5, 100 mM NaCl, 50 mM NaF, supplemented with mammalian protease inhibitor cocktail and phosphatase inhibitor cocktail II; Sigma-Aldrich, St. Louis, MO). Lysates were precleared by centrifugation, and 150–300 μ g of each was rotated end over end for either 2 h at 4°C with 3 μ l of anti-Met^{Hu} intracellular domain antibody (sc-10) or

4 h at 4°C with 4 μ l of anti-Hrs antibody, together with protein A-Sepharose (Amersham Biosciences AB, Uppsala, Sweden). Immunoprecipitates (IPs) were washed three times in 0.1% NP-40 (Merck, Leicestershire, United Kingdom), 25 mM Tris-HCl (pH 7.5), and 150 mM NaCl supplemented with phosphatase inhibitor cocktail II and then once in 10 mM Tris-HCl (pH 7.5) before preparation for SDS-PAGE. After SDS-PAGE on 8% gels, proteins were then transferred to polyvinylidene difluoride membrane (0.45- μ m pore size; Schleicher & Schuell, Keene, NH) and then blocked overnight at 4°C by incubation in blocking buffer (1% bovine serum albumin [BSA], 0.1% Tween 20 [Merck] in 10 mM Tris-HCl pH 7.5, 100 mM NaCl). Primary and secondary antibody incubations were for 1 h at room temperature in blocking buffer. Proteins were detected via enhanced chemiluminescence (ECL) (Pierce Chemical, Rockford, IL), and resultant Western blots were stripped and reprobed using anti-Met^{tr} intracellular domain antibody (sc-10) or anti-Hrs accordingly, to assess the amount of Met/Hrs immunoprecipitated in each sample.

To assess HGF/SF-dependent phosphorylation of mitogen-activated protein kinase kinase (MEK), equal quantities of lysate were resolved on 12% gels by SDS-PAGE, transferred to nitrocellulose membrane (0.45- μ m pore size; Schleicher & Schuell), and blotted using anti-phospho-MEK1/2 antibody according to the manufacturer's instructions. Blots were then stripped and reprobed to assess parallel MAP kinase phosphorylation by using anti-phospho-p44/42 MAP kinase antibody, also according to the manufacturer's instructions.

HGF/SF-dependent Met Ubiquitination Assay

Cells were stimulated ($\pm$ proteasome inhibitors) and lysed as described under "Cell Signaling Assay." Lysates were precleared by centrifugation, and 200–400 μ g of each was mixed overnight with either 1.5 μ l of anti-Met^{tr} extracellular domain antibody (05–237) plus protein A-Sepharose, or 1 μ l of antibody to protein-ubiquitin conjugates (FK2) plus protein G-Agarose (Sigma-Aldrich). The next day, IPs were washed and resolved by SDS-PAGE as described above. After transfer to nitrocellulose, membranes were blocked overnight at 4°C in blocking buffer. Transferred anti-Met^{tr} IPs were probed with FK2 antibody, and transferred FK2 IPs were probed with anti-Met^{tr} intracellular domain antibody (sc-10) followed by ECL.

Cell-Surface Biotinylation and Met Internalization/Recycling Assay

Cells were seeded in medium containing serum and allowed to adhere to the dish, after which they were starved for 16–20 h in serum-free medium. When required, cells were preincubated in either lactacystin (for 2 h) or *clasto*-lactacystin β -lactone (for 1 h) before being rapidly chilled on ice for biotinylation of cell surface proteins as described previously (Altschuler *et al.*, 2000). Briefly, cells were washed three times with ice-cold PBS and incubated with 0.25 mg/ml sulfo-succinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (EZ-Link Sulfo-NHS-SS-Biotin; Pierce Chemical) in 150 mM Na₂B₄O₇ pH 8 for 15 min on ice. The reaction was quenched by washing the cells three times with ice-cold serum-free media containing 0.1% BSA and then once with ice-cold HEPES-buffered saline (HBS; 10 mM HEPES-NaOH pH 7.4, 150 mM NaCl) containing 0.7 mM CaCl₂ and 0.5 mM MgCl (HBS⁺⁺). Cells were then stimulated by adding prewarmed (to 37°C) serum-free medium containing ~250 ng/ml HGF/SF ($\pm$ proteasome inhibitors, where indicated) and rewarmed to 37°C for the desired time period(s) to allow internalization of the biotinylated surface components, after which they are returned to 4°C. After one rinse with HBS⁺⁺, biotin remaining at the cell surface was stripped by three 20-min incubations with ice-cold 100 mM sodium 2-mercaptoethanesulfonic acid (MESNA) in 50 mM Tris-HCl pH 8.6, 100 mM NaCl, 1 mM EDTA, and 0.2% BSA. The cells were then quickly rinsed twice again with HBS⁺⁺, and residual MESNA was quenched by a 10-min incubation

with ice-cold 120 mM iodoacetamide in HBS⁺⁺. After two additional rinses with ice-cold HBS cells were solubilized at room temperature with 60 mM *n*-octyl β -D-glucopyranoside and 0.1% SDS (both from Sigma-Aldrich) in HBS containing protease inhibitor cocktail for 10–20 min, and insoluble material was removed by centrifugation in a microcentrifuge at 15,000 rpm for 7 min.

Supernatants were recovered and 150 μ g of each rotated end over end overnight at 4°C with 40 μ l of immobilized NeutrAvidin (Pierce Chemical). Biotin-NeutrAvidin precipitates were recovered by brief centrifugation and washed three times with 1% Triton X-100 (Merck) in HBS, and then once with HBS before being prepared for SDS-PAGE. After SDS-PAGE on 8% gels, proteins were transferred to nitrocellulose then blocked by incubation in PBS containing 5% nonfat dry milk (blocking buffer). Membranes were incubated with anti-Met^{tr} intracellular domain antibody (sc-10) in blocking buffer for at least 1 h (at room temperature) or overnight (at 4°C). Proteins were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibody (1-h incubation at room temperature) followed by ECL.

For experiments investigating Met recycling, the protocol was modified slightly to avoid stressing cells by keeping them at 4°C for long periods. After cell-surface biotinylation and 10-min HGF/SF stimulation, remaining surface biotin was stripped by three 10-min incubations in the aforementioned MESNA solution followed by an iodoacetamide quench. Cells were then rewarmed to 37°C in serum-free medium but without ligand ($\pm$ lactacystin) for either 15 or 30 min, to allow recycling of internalized biotinylated Met molecules. This was followed by three additional 10-min MESNA incubations, again at 4°C, and subsequent iodoacetamide quench after which the cells were solubilized for analysis as described above.

Depletion of Cellular Hrs by Short Interfering RNA (siRNA) Treatment

HeLa cells were seeded in medium containing serum and allowed to adhere overnight. The medium was replaced with DMEM (without serum and antibiotics), and the cells were transfected with 1.8 nmol (per 1.54×10^6 cells) of siRNA duplex (sense, GUCAACGA-CAAGAACCCACdTdT; antisense, dTdTTCAGUUGCUGUUCU-UGGGTG; [Dharmacon, Lafayette, CO]) by using oligofectamine (Invitrogen), according to the manufacturer's protocol. Four hours posttransfection, fetal bovine serum was added to the cells, giving a final serum concentration of 7.5%. Fifty-six hours posttransfection, the cells were detached from the dish by a 5-min incubation in 2 ml of trypsin-EDTA and reseeded in DMEM containing 10% fetal bovine serum. The following day (72 h posttransfection), cells were stimulated with 250 ng/ml HGF/SF and then washed several times with ice-cold PBS and lysed for 15 min on ice in TNTE lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.3% [wt/vol] Triton X-100, 5 mM EDTA, supplemented with mammalian protease inhibitor cocktail) or NP-40 lysis buffer (0.5% NP-40, 25 mM Tris pH 7.5, 100 mM NaCl, 50 mM NaF, supplemented with mammalian protease inhibitor cocktail). Lysates were precleared by centrifugation and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with anti-Hrs to determine the levels of Hrs remaining. Blots were routinely reprobed with anti-tubulin to confirm equal loading of lysate proteins.

RESULTS AND DISCUSSION

Proteasome Activity and Endosomal Trafficking of Met

We have adapted an assay for internalization of cell surface molecules to directly measure Met endocytosis after acute application of HGF (Schmid and Smythe, 1991; Altschuler *et al.*, 2000). Cell surface proteins are labeled at 4°C with a biotin moiety that contains a disulfide bond and hence are

Figure 1. Isolation of biotinylated Met: efficiency of MESNA reduction and optimization of cell-surface Met biotinylation. (A) HeLa cells were incubated on ice for 15 min $\pm$ 500 μ g/ml disulfide-cleavable biotin, as described in MATERIALS AND METHODS. The cells were then washed of excess biotin by three quenching rinses and either lysed immediately (lane 2), or after three incubations in a solution containing the reducing agent MESNA (lane 3), plus additional washes. Surface biotinylated molecules were recovered with NeutrAvidin-agarose beads, resolved by 8% SDS-PAGE, and examined by Western blot analysis under reducing conditions using anti-Met^{Hu} intracellular-domain antibody. MESNA efficiently cleaves all biotin-Met moieties. (B) Biotinylations were carried out with varying concentrations of biotin. Excess biotin was removed by three quenching rinses and the cells were then lysed. i) Surface biotinylated molecules were recovered with NeutrAvidin-agarose beads, resolved by SDS-PAGE, and examined by Western blot analysis under reducing conditions using anti-Met^{Hu} intracellular-domain antibody. ii) The unbound fraction from each precipitation reaction was also probed with the same anti-Met^{Hu} antibody. Molecular mass markers (in kilodaltons) are shown on the left. Using 250 μ g/ml or more of biotin (lanes 5 and 6) results in only a very small (<5%) p140^{Met} signal in the unbound fraction, implying that a high proportion of total mature Met, under steady-state conditions, resides at the plasma membrane and is accessible to biotinylation at these concentrations.

cleaved by exposure to reducing agents. After return to 37°C medium, containing HGF, biotin-Met is sequestered into internal compartments and is thereby protected from cleavage by subsequent application of an impermeable reducing agent (MESNA). Control experiments verified that the MESNA stripping procedure was 100% efficient (Figure 1A). The optimal concentration of biotinylation reagent for surface Met labeling was determined by titration (Figure 1B). Increased recovery of biotin-Met bound to NeutrAvidin-agarose with increasing biotinylation reagent is mirrored by depletion of total mature Met from the unbound fraction, whereas the intracellular higher molecular weight precursor form (p170^{Met}) does not bind to NeutrAvidin-agarose. After biotinylation, >95% of total mature Met is depleted and must therefore be cell surface associated during the labeling procedure at 4°C (Figure 1B, bottom). In unstimulated HeLa cells, nearly all mature Met is cleaved after application of MESNA. After HGF stimulation, a significant proportion of biotin-Met becomes protected from MESNA within 5 min, indicating that Met is rapidly internalized in response to ligand binding. Early time points of this assay should purely reflect Met internalization rather than a composite of path-

Figure 2. Inhibition of the proteasome has no effect on ligand-induced Met internalization but instead promotes recycling to the plasma membrane. (A) Cell-surface receptors were biotinylated on ice with a disulfide-cleavable biotin as described in MATERIALS AND METHODS. Cells were then rapidly rewarmed to 37°C and cultured in medium with or without HGF/SF and/or proteasome inhibitor for the indicated times to allow internalization of surface Met, after which they were rapidly cooled on ice, and remaining cell-surface biotin stripped with MESNA. After lysis, internalized biotinylated molecules were recovered with NeutrAvidin-conjugated beads, resolved by SDS-PAGE, and examined by Western analysis under reducing conditions using anti-Met^{Hu} intracellular-domain antibody. Similar results were obtained in three experiments. (B) To investigate recycling of Met after a 10-min HGF/SF "pulse," cells were rewarmed after MESNA stripping, but in the absence of ligand, for 15 or 30 min at 37°C then subjected to a second round of MESNA stripping before lysis and analysis as described in A. Molecular mass markers are shown on the left.

ways, because neither significant degradation of Met nor recycling back to the plasma membrane will take place in this time window. Within the experimental error of the assay we could not detect a significant effect of lactacystin on the extent or kinetics of internalization (Figure 2A). For quantitation of early time points from internalization experiments as shown in Figure 2A, each biotin-Met band was ratioed against a corresponding tubulin band from the cell lysate from which it was derived. Intensity ratios were normalized to the signal obtained after 10-min internalization in the absence of lactacystin (set to 100). At time 3 min after HGF application the intensity value was 3.9 ± 1 in the absence of lactacystin and 2.9 ± 1.4 in the presence of lactacystin ($n = 4$). At time 5 min, these values increase respectively to 18.9 ± 4.5 and 17.8 ± 6.9 ($n = 4$). Our previous observation that lactacystin treatment confines Met to the plasma membrane at later time points (Hammond *et al.*, 2001) must therefore reflect enhanced recycling to the plasma membrane at the expense of sorting toward late endosomes. This is confirmed by a further experiment, which examines the ability of internalized biotin-Met to return to the cell surface where it will become sensitive to a second MESNA treatment. In the absence of lactacystin, essentially no biotin-Met returns to the cell surface after HGF-stimulated internalization, whereas in the presence of lactacystin, biotin-Met is recycled to the cell surface (Figure 2B). Relative to their respective controls, the biotin-Met signal is consequently reduced after chase periods of 15 min (to

Figure 3. Ligand-induced ubiquitination of Met can occur in the presence of proteasome inhibitors, although subtle differences in the pattern of ubiquitination are apparent. HeLa cells were cultured in medium with or without HGF/SF as indicated. Equal quantities of lysates from cells harvested at the indicated times after HGF/SF addition were immunoprecipitated, resolved by SDS-PAGE on 5% or 8% gels, and examined by Western analysis under reducing conditions by using the indicated antibody combinations. Molecular mass markers are shown on the left. Similar results were obtained in five experiments.

$69.5 \pm 9.1\%$, $n = 3$, $p < 0.05$) and 30 min (to $64.2 \pm 3.6\%$, $n = 3$, $p < 0.005$) in the presence of lactacystin. The similar levels of surface Met observed between 15- and 30-min chase period (+lactacystin) indicates that after a 10-min acute application of HGF, a new steady-state distribution of Met is achieved within 15-min subsequent chase period. Our failure to observe inhibition of internalization now excludes a proposal that Met shares a proteasome activity-dependent internalization mechanism with the GHR (van Kerkhof *et al.*, 2000).

Met Ubiquitination and Lactacystin

Ubiquitination has recently been identified as a requirement for the targeting of several proteins to internal luminal vesicles of endosomes (Katzmann *et al.*, 2001; Reggiori and Pelham, 2001; Urbanowski and Piper, 2001), and indeed the Met receptor is ubiquitinated after stimulation (Jeffers *et al.*, 1997b). It is possible that proteasome inhibition by lactacystin may affect Met trafficking indirectly. In particular, it could interfere with receptor ubiquitination by preventing the recycling of cellular ubiquitin and consequently reducing cellular levels of free ubiquitin. We have analyzed both HGF-dependent ubiquitination of Met after immunoprecipitation with antibodies against Met, and the incorporation of Met to an anti-ubiquitin immunoprecipitate. Both experiments reveal that HGF-dependent ubiquitination can occur after incubation with lactacystin treatment but at slightly reduced levels (Figure 3).

Minor differences, due to lactacystin, in the ubiquitination pattern are nevertheless apparent; for example, a high-molecular-weight ubiquitinated band is more apparent in immunoprecipitates of Met from lysates of lactacystin-treated cells. We consider it unlikely that the observed differences may impose the virtually complete block to HGF-dependent

Met degradation that we see with lactacystin, but it cannot be completely discounted. Alternatively, obligate ubiquitination of an accessory factor may be more sensitive to lactacystin, and this factor may even have to be degraded for efficient Met sorting.

Incorporation into luminal vesicles is not compulsory for late endosomal targeting. Several proteins that are normally sorted into these internal vesicles contain sorting information that allows their advancement to the limiting membrane of late endosomes/lysosomes (or the yeast vacuole) when the luminal sorting machinery is defective. This is in fact the case for epidermal growth factor receptor (EGFR) when luminal vesicle formation is inhibited by the phosphatidylinositol 3-kinase inhibitor wortmannin (Futter *et al.*, 2001). EGFR is degraded after transport to the limiting membrane of late endosomes/lysosomes. This also seems to hold true when luminal vesicle incorporation of EGFR is blocked by lactacystin (Longva *et al.*, 2002). Our previous results indicate that Met must lack the sorting information to take this alternative route in the presence of lactacystin. If we assume there is a common factor in the requirement of proteasome activity for luminal sorting of EGFR and Met, then a deficit of alternative sorting information may explain the preferential sensitivity to lactacystin of Met degradation.

Endosome Sorting and Signaling Outputs

We have examined the effect on Met signaling of perturbing its trafficking itinerary by lactacystin. Note that the foregoing results and discussion would argue against any effect due to defective internalization, and more likely reflect partitioning within the sorting endosome and perhaps as a consequence residence time at that compartment.

Acute stimulation with HGF leads to receptor autophosphorylation that normally peaks within 10 min and subsequently declines (attenuation). In HeLa cells used throughout this study, the kinetics is such that the dephosphorylation can be dissociated from receptor degradation as evidenced by a decline in the ratio of phospho-Met to total Met (Figure 4A). Under the cell culture conditions used in these experiments, there is little degradation of Met within the first 30 min post-HGF stimulation (see Figure 1 of Hammond *et al.*, 2001). In cells preincubated with lactacystin, this attenuation of Met phosphorylation is inhibited with levels remaining at peak values for over an hour.

Lactacystin could directly affect the dephosphorylation of Met, or this effect could be due to its influence on Met dynamics. We favor the latter hypothesis because inhibition of Met internalization by expression of dominant negative endophilin (Petrelli *et al.*, 2002) or dynamin (Figure 4C) also inhibits the attenuation of phospho-Met. Thus, not only would internalization be required for Met dephosphorylation but also correct partitioning within or retention at the sorting endosome.

One of the best-characterized pathways downstream of Met activation is the MAP kinase pathway (Furge *et al.*, 2000; Karihaloo *et al.*, 2001; Tsukada *et al.*, 2001). We have analyzed the HGF-dependent phosphorylation profile of extracellular signal-regulated kinase (ERK) and of its upstream partner MEK. Both the initial response and subsequent attenuation is insensitive to lactacystin treatment (Figure 5). Thus, somewhat counterintuitively, MAP kinase attenuation

Figure 4. Proteasome activity and clathrin-mediated endocytosis are required for the dephosphorylation of Met that occurs after an HGF/SF-induced phosphorylation. (A) HeLa cells were preincubated for 2 h with (+) or without (–) lactacystin and then stimulated with HGF/SF for the times indicated. The cells were then lysed and proteins immunoprecipitated with anti-Met antibody. Immunoprecipitated proteins were analyzed by immunoblotting with PY20 antibody (top) or anti-Met (bottom). (B) Intensity of phospho-Met bands (top) and total Met bands (bottom) was quantitated using NIH Image 1.62 software and a ratio of phospho-Met:Met was calculated for each time point. Phospho-Met:Met after 30-min stimulation was expressed as a percentage of phospho-Met:Met after 10-min stimulation. Each data point represents mean $\pm$ SEM of five different experiments. * $p < 0.005$. (C) K44A cells were cultured for 48 h with (+) or without (–) tetracycline and then stimulated with HGF/SF for the times indicated. Analysis of Met phosphorylation was performed as in A.

proceeds in the face of sustained Met activation. The relevant phosphatase is thus most likely freely diffusible, its activation lagging behind MAP kinase activation but also sustaining this activity to suppress the MAP kinase response to continued Met activation.

Hrs is an endosome-localized substrate for activated tyrosine kinase receptors that is involved in receptor sorting (Komada and Soriano, 1999; Takata *et al.*, 2000; Komada and Kitamura, 2001; Clague, 2002). In the case of EGF stimulation, dynamin-dependent endocytosis (presumably of receptor) must occur for efficient Hrs phosphorylation (Urbé *et al.*, 2000). We now confirm that this is also the case after HGF

stimulation by using withdrawal of tetracycline from K44A cells to induce expression of dominant negative dynamin (Figure 6A). Lactacystin treatment also dramatically inhibits HGF-dependent phosphorylation of Hrs. Conversely, EGF-dependent phosphorylation of Hrs is not reduced after lactacystin treatment but is even relatively increased at late time points (Figure 6, B and C). Hrs is highly enriched within a distinctive bilayered clathrin-containing coat that decorates the surface area of sorting endosomes and is also a site of concentration of receptors destined for late endosomes (Sachse *et al.*, 2002). In neither case is lactacystin blocking internalization of receptor; we therefore propose that lactacystin is differentially affecting access or retention in this coated region and hence access to its substrate Hrs. Phosphorylation of Hrs would then reflect, and may even be coupled to, correct endosomal sorting of tyrosine kinase receptors. Formal proof will require electron microscopic quantitation of Met distribution in endosomes, but our preliminary efforts in this direction with available antibodies have not yielded high enough labeling efficiencies. A precedent for lactacystin inhibition of receptor sorting into these clathrin-coated areas is provided from the study of Sachse *et al.* (2002) wherein a truncated form of GHR is shown to fail to concentrate in these areas after lactacystin treatment.

Hrs and its yeast ortholog Vps27 have been implicated in sorting of receptors at early endosomes toward late endosomes/prelysosomal compartments (Piper *et al.*, 1995;

Figure 5. HGF/SF-dependent phosphorylation profiles of ERK and MEK are insensitive to lactacystin. HeLa cells were preincubated for 2 h with (+) or without (–) lactacystin and then stimulated with HGF/SF for the times indicated. Cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phospho-ERK1/2 (top) and anti-phospho-MEK1/2 (bottom).

Figure 6. Proteasome activity and clathrin-mediated endocytosis modulate HGF/SF and EGF-induced Hrs phosphorylation. (A) K44A cells were cultured for 48 h with (+) or without (–) tetracycline and then stimulated with HGF/SF for the times indicated. The cells were then lysed and proteins immunoprecipitated with anti-Hrs antibody. Immunoprecipitated proteins were analyzed by immunoblotting with PY20 antibody (top) or anti-Hrs (bottom). (B and C) HeLa cells were preincubated for 2 h with (+) or without (–) lactacystin and then stimulated with HGF/SF or EGF for the times indicated. Analysis of Hrs phosphorylation was performed as in A. (D) Intensity of phos-

Figure 7. HRS knockdown by siRNA retards HGF-dependent degradation of Met and inhibits dephosphorylation. HeLa cells were treated with siRNA, targeted against Hrs for 72 h, or underwent a dummy transfection protocol as described in MATERIALS AND METHODS. Cells were then stimulated with HGF for the indicated times. Cell lysates were blotted with anti-Met, anti-PY1349-Met (which selectively recognizes a phosphotyrosine containing peptide that provides a docking site for proteins downstream of Met activation), anti-Hrs, and anti-tubulin, as indicated.

Takata *et al.*, 2000; Urbé *et al.*, 2000; Komada and Kitamura, 2001; Raiborg *et al.*, 2002). We have depleted cellular levels of Hrs by using short interfering RNA treatment (Elbashir *et al.*, 2001), which depletes >90% of Hrs within 72 h (levels are reduced to ~13 and 6% at 48 and 72 h, respectively). Analysis was complicated by the fact that the cell culture conditions adopted for these experiments induced a rapid HGF-dependent Met down-regulation component (within 10 min) even in control cells, not seen under our routine cell culture conditions. We have not pursued the nature of this pathway further in this study, although it would seem to be too rapid to represent a lysosomal pathway. Nevertheless, at later time points after this initial HGF-dependent drop in Met levels, we observed a modest, but by no means complete, retardation in HGF-dependent down-regulation of Met (Figure 7A, compare 60-min time points). It is noteworthy that previous studies implicating Hrs in EGF receptor trafficking have relied on overexpression of Hrs being dominant negative (perhaps due to titration of accessory factors). These observations have been open to several interpretations, as overexpression of Hrs has a profound influence on the or-

pho-Hrs bands (top) and total Hrs bands (bottom) from C were quantitated using NIH Image 1.62 software and a ratio of phospho-Hrs:Hrs was calculated for each time point. Phospho-Hrs:Hrs after 20-min stimulation was expressed as a percentage of phospho-Hrs:Hrs after 8-min stimulation. Each data point represents mean $\pm$ SEM of three experiments. * $p < 0.05$.

ganization of the endocytic system (Urbé *et al.*, 2000; Raiborg *et al.*, 2001; Bishop *et al.*, 2002). Our results concur with an Hrs knockout study in *Drosophila*, for which degradation of EGF receptor and *Torso* tyrosine kinase receptor were shown to be defective (Lloyd *et al.*, 2002).

We reasoned that because Hrs depletion exerts a similar effect to lactacystin on Met degradation, perhaps there also would be a parallel failure of Met dephosphorylation. Data shown in Figure 6 indicate that this is indeed the case. In the example shown, we have used a phospho-specific Met antibody that recognizes a peptide containing phospho-Tyr (1349) that provides a docking site on activated Met for downstream factors. This has the advantage that cell lysates can be blotted directly rather than having to first immunoprecipitate and then blot with PY20 antibody, although similar results were obtained with both approaches. After siRNAi depletion of Hrs, HGF-dependent Met phosphorylation fails to attenuate from its maximal value to the same extent that is seen with control cells. This observation reinforces the findings obtained with lactacystin treatment that correct partitioning within sorting endosomes, probably to bilayered clathrin coated areas, facilitates both Met dephosphorylation and degradation.

CONCLUSION

We have established an assay that allows the biochemical determination of HGF-dependent endocytosis of Met. This has allowed us to establish that a lactacystin-imposed block to Met endocytic trafficking does not reflect a failure to internalize Met but rather enhanced recycling to the plasma membrane at the expense of sorting to late endosomal compartments. In this article, we have described three signaling outputs of HGF stimulation that show varying responses to lactacystin, elevation/prolongation (phospho-Met), no significant change (MAP kinase) or significant reduction (Hrs). This adds to an already substantial body of work linking receptor dynamics to signaling, but whereas those previous studies have largely relied upon a block to receptor internalization, we show that Met internalization is not inhibited by lactacystin treatment. However, for HGF stimulation, the effects of lactacystin, which promotes recycling from the sorting endosome, on the three signaling outputs that we have examined, mirror the effects of blocking receptor internalization. A deviation from this rule can be seen with EGF-dependent Hrs phosphorylation, which is inhibited by blocking internalization (Urbé *et al.*, 2000) but is sustained by lactacystin treatment.

We conclude that receptor interactions within the sorting endosome are key determinants of signaling outcome. Perhaps this could be anticipated when one considers the paradigmatic example of the EGFR, for which it can be estimated that at steady state, after acute stimulation, up to 80% of activated receptors are endosomally localized (Sorkin, 1998).

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