Protein Kinase C Controls Microtubule-based Traffic but Not Proteasomal Degradation of c-Met*

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Upon hepatocyte growth factor stimulation, its receptor c-Met is rapidly internalized via clathrin-coated vesicles and traffics through an early endosomal compartment. We show here that c-Met accumulates progressively in perinuclear compartments, which in part include the Golgi. The c-Met content in the Golgi is principally the newly synthesized precursor form and, to a lesser extent, the internalized, recycling c-Met. By following the trafficking of c-Met inside the cell using a semi-automatic procedure and using inhibition or activation of protein kinase C (PKC) and microtubule depolymerizing agents, we show that PKC positively controls the trans-cytosolic movement of c-Met along microtubules. In parallel to its traffic, internalized c-Met is progressively degraded by a proteasome-sensitive mechanism; the lysosomal pathway does not play a substantial role. Inhibition or promotion of c-Met traffic to the perinuclear compartment does not alter the kinetics of proteasome-dependent c-Met degradation. Thus susceptibility to proteasomal degradation is not a consequence of post-endocytic traffic. The data define a PKC-controlled traffic pathway for c-Met that operates independently of its degradative pathway.

The tyrosine kinase receptor c-Met is the high affinity receptor for hepatocyte growth factor (HGF).1 Signaling via this receptor-ligand pair can induce diverse biological events. In vitro, these include scattering, invasion, proliferation, branching morphogenesis, and angiogenesis. In vivo, it is responsible for many processes during embryonic development and a variety of activities in the adult; many of these normal activities have been implicated in its role in tumorigenesis and metastasis (reviewed in Refs. 1 and 2). Overexpression of c-Met has been observed in a large number of human tumors, correlating closely with metastatic tendency and poor prognosis (3–6). Furthermore, germ line missense mutations of c-Met, which lead to increased tyrosine kinase activity, have been reported in childhood hepatocellular carcinoma (7). A molecular understanding of how this receptor is switched on and off will provide the basis for developing rational interventions in such situations.

C-Met, encoded by the c-met proto-oncogene is a disulfide-linked α/β heterodimer, derived by proteolytic processing of the precursor p170met (8). The β-chain, p145met, spans the plasma membrane and includes a cytoplasmic domain endowed with tyrosine kinase activity. The mechanisms by which c-Met triggers its regulatory functions involve the activation of several intracellular signaling pathways through a unique multisubstrate docking site within the C-terminal end of the receptor (reviewed in Ref. 9).

It is well established that many transmembrane receptors become internalized upon ligand stimulation. Although EGFR endocytosis and traffic have been extensively studied and represents probably the best understood receptor trafficking system (10), interest in c-Met and its traffic are just emerging. Several receptors such as EGFR or platelet-derived growth factor receptor are ubiquitinated and then degraded by the lysosomal pathway (reviewed in Refs. 11 and 12). So far, it has been reported that c-Met is polyubiquitinated (13) via the c-Cbl proto-oncogene (14), (15) and degraded in a proteasome-dependent manner (13). However, more recent studies (16) indicate that the primary effect of proteasome inhibition on c-Met could occur indirectly through an effect on acute HGF-induced c-Met endocytic traffic. Similar results have been described for growth hormone receptor (17).

In view of the critical role played by c-Met in cancer, it is important to know how its endocytosis and trafficking are regulated. The endocytosis of several membrane receptors is activated by PKC. For example this is the case for the γ-aminobutyric type A receptor, the parathyroid hormone receptor 1, and the σ2A somatostatin receptor (18–20). Prior evidence indicates that PKC plays a negative role in controlling c-Met function (21), however it is not known how this relates to the internalization or traffic of c-Met.

In this study we show that, upon HGF stimulation, the rapidly internalized c-Met traffics and accumulates in a perinuclear compartment, which in part includes the Golgi, corresponding presumably to a recycling compartment. By following the trafficking of c-Met inside the cell using a semi-automatic assessment of vesicle distribution, we demonstrate that this trans-cytosolic movement of c-Met requires an intact microtubule network and is promoted by PKC. After 2 h of HGF stimulation, half of the internalized c-Met has been degraded by a proteasomal pathway. C-Met is likely a direct target of the proteasome, because we do not detect any effect of inhibition of proteasome activity on internalization of c-Met; furthermore, the lysosomal pathway does not play a substantial role. Inhibition or promotion of c-Met traffic to the perinuclear compartment does not alter the kinetics of proteasome-dependent c-Met degradation. Thus susceptibility to proteasomal degradation follows receptor internalization but is not a consequence of post-endocytic traffic.
EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Cancer Research UK) supplemented with 10% fetal bovine serum (Sigma) and maintained at 37 °C in a humidified 10% CO₂ atmosphere. The cells were seeded in 35-mm plates (for Western blot experiments) or on coverslips in 24-well plates (for immunocytochemistry) and stimulated 24 h later with 100 ng/ml HGF for various time periods. Where indicated, the cells were preincubated with appropriate inhibitors 10 or 15 min before HGF stimulation, and all the inhibitors were maintained during the stimulations. The preincubation times were routinely longer for proteasome inhibitors; 2 h for lactacystin and 1 h for MG132; shorter preincubations are as indicated in the text or figure legends.

Transfections were performed on cells seeded on coverslips in 24-well plates. For each well, 0.8 μg of DNA was mixed with 1.5 μl of LipofectAMINE™ 2000 (Invitrogen) in 100 μl of OPTIMEM1 with Glutamax (Invitrogen), incubated at room temperature for 20 min to allow the precipitate to form and directly added to the cells in their culture medium. Five hours later the culture medium was changed. Stimulation was performed 24 h post-transfection.

Growth Factors, Antibodies, Inhibitors, and Constructs—Purified human recombinant HGF was obtained from R&D Systems. The following antibodies were used: affinity-purified rabbit polyclonal anti-human c-Met intracellular domain (12-amino acid CT, Santa Cruz Biotechnology); goat polyclonal anti-early endosome autoantigen 1 (EEA1; Santa Cruz Biotechnology); mouse monoclonal antibodies anti-tubulin (Santa Cruz Biotechnology for Western blots and Sigma for immunofluorescence); mouse monoclonal anti-c-Met extracellular domain (Upstate Biotechnology) and mouse monoclonal antibodies against BM12/GM130 (BD Biosciences); goat polyclonal anti-human EGFR (Santa Cruz Biotechnology); goat anti-human HGF (Sigma); mouse monoclonal anti HGF (R&D System). The secondary antibodies used for Western blot were peroxidase-labeled monkey anti-mouse or anti-rabbit IgG (Dako). The secondary antibodies used for immunofluorescence; mouse monoclonal anti-c-Met extracellular domain (12-amino acid CT, Santa Cruz Biotechnology); goat anti-human EGFR (Santa Cruz Biotechnology); goat anti-human HGF (Sigma); mouse monoclonal anti HGF (R&D System). The secondary antibodies used for Western blot were peroxidase-labeled monkey anti-mouse or anti-rabbit IgG (Dako). The secondary antibodies used for immunofluorescence were as follows: Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes), cy3- or cy5-conjugated affinity-purified donkey anti-mouse IgG and cy5-conjugated affinity-purified donkey anti-goat IgG (Jackson ImmunoResearch). MG132, lactacystin, concanamycin, bafilomycin A1, paclitaxel (or taxol), viablinsulfate were obtained from Calbiochem. Nocodazole, colchicine, cycloheximide, propidium iodide, TPA (phorbol-12-myristate 13-acetate), and RNase A were purchased from Sigma. Bisindolylmaleimide I (BIM-I) was obtained from Alexix Biochemicals. LysoTracker was obtained from Molecular Probes. The pCMV-Myc r-AP180 C terminus (residues 530–915) (myc-AP180-C) construct was a generous gift from Dr. Harvey T. McMahon (Cambridge, UK).

Immunofluorescence and Confocal Analysis—Cells were washed twice in PBS and fixed in 2% paraformaldehyde for 10 min. Free aldehydes were quenched with 50 μl NH₄Cl in PBS for 10 min. Fixed cells were permeabilized in 0.1% Triton X-100 in PBS-2% bovine serum albumin for 15 min. For microtubule staining, the fixation and permeabilization were as follows: cells were incubated for 30 s in a buffer containing 1% Nonidet P-40, 100 mM Pipes, 2 mM EDTA, 1 mM MgCl₂, and 0.1 mM EGTA. They were then immediately incubated in methanol at −20 °C for 2 min. Fixed cells were incubated at room temperature for 1 h with the primary antibodies at the following concentrations: anti-c-Met (1 μg/ml), anti-EEA1 (2 μg/ml), anti α-tubulin (1/2000), anti-p-115 (1/2000), and anti-GM130 (1/200). Cells were rinsed and incubated with appropriate secondary antibodies (5 μg/ml) for 30 min. When indicated, nuclei were stained as follows: cells were incubated in RNase A (1 μg/ml) for 15 min and then in propidium iodide (0.2 μg/ml) for 10 min. Cells were washed three times in PBS and once in water and then mounted in Mowiol containing 2.5% DABCO (1,4-diazabicyclo[2.2.2]octane). Images were acquired using a confocal laser scanning microscope (LSM510, Carl Zeiss Inc.) equipped with a 63×/1.4 Plan-Apochromat oil immersion objective. Alexa 488 was excited with the 488-nm line of an argon laser, Cy3 was excited with a 543-nm HeNe laser and Cy5 was excited with a 633-nm HeNe laser. Each image represents a single section.

Western Blot Analysis—Cells were harvested in 100 μl of Laemmli sample buffer (22) and boiled for 10 min. Samples were loaded on 7.5% polyacrylamide gels. Separated proteins were transferred to a 0.45-μm nitrocellulose transfer membrane (Schleicher & Schuell). Protein loading was checked by staining with Ponceau Red. Membranes were then blotted with the intracellular domain c-Met antibody and anti-α-tubulin. Blots were then incubated with appropriate peroxidase-linked secondary antibodies. Immunoblots were revealed using the ECL Western blotting detection reagents (Amer sham Biosciences).

Semiautomatic Assessment of Vesicles in the Cytosol—Fluorescence images of cell nuclei and vesicles were acquired on an Axiosvert TM 135 microscope (Carl Zeiss) equipped with a 63× numerical aperture 1.3 objective lens and an Orca ER CCD camera (Hamamatsu) using Acquisition Manager (Kinetic Imaging). Multiple dichroic with excitation and emission filter wheels ensured that there was no lateral shift between the two fluorescent channels. Cell nuclei were automatically detected by thresholding, and their boundaries were interpolated by elliptical curves (see Fig. 1B). c-Met-positive vesicles were detected by Sobel image enhancement followed by thresholding. Cell boundaries were interactively determined. Two distances were calculated for each vesicle: the distance from the cell boundary (a) and the distance to the nuclear boundary (b) along a line passing through the center of the nucleus elliptical boundary. The relative distance of a vesicle from the cell boundary was evaluated as a/a + b. Relative distances can have values between 0 (vesicle at the cell boundary) and 1 (vesicle at the nucleus). The relative distances were weighted according to the total intensity of fluorescence in the vesicles for calculations of mean values, standard deviations, and analysis of variance (ANOVA) with a hierarchical unbalanced model. The following levels and total numbers of data were used in the hierarchical structure: relative distances (78,637), cells (976), observation fields (357), cell cultures (67), experiments (40), and treatments (12).

RESULTS

c-Met Is Rapidly Internalized upon HGF Stimulation and Traffics to a Perinuclear Compartment—In the absence of ligand, c-Met is predominantly distributed around the plasma membrane with some asymmetrically distributed, punctate, perinuclear staining (Fig. 1A, top left image). HGF treatment of HeLa cells leads to the rapid endocytosis of c-Met with the receptor transiently accumulating on vesicular structures, followed by a delayed transcytotic traffic to and accumulation within a perinuclear compartment. We developed a semiautomatic procedure, as described under “Experimental Procedures,” to follow the migration of fluorescent c-Met vesicles, from the plasma membrane to this perinuclear location (Fig. 1B). This indicated that following internalization the bulk of the immunoreactive protein accumulated within the perinuclear compartment between 30 and 120 min. The initial endocytic event is typical of a clathrin-mediated process, being inhibited by co-transfected myc-AP180-C (Fig. 1C) and by pretreatment with dansylcadaverine (Fig. 1D). Contrary to previous studies (16) no inhibition of endocytosis was observed on treatment with the proteasome inhibitors MG132 or lactacystin (Fig. 1E; see further below). At 15 min, the internalized c-Met is found to overlap with EEA1 indicative of an early endocytic compartment; this co-localization has decreased by 120 min (Fig. 1F). HGF co-localizes with c-Met in vesicles and is still present after 120 min of treatment (Fig. 1G). This co-localization has been detected with two distinct HGF-specific antibodies, a polyclonal and a monoclonal antibody.

PKC Controls the Movement of c-Met from Early Endosome to a Perinuclear Compartment—It has been shown that PKC can negatively influence c-Met signaling (21, 23). To establish whether this action is effected through the control of HGF-induced traffic of c-Met, an inhibitor of PKC, BIM-I (bisindolylmaleimide I) was employed. BIM-I did not have a substantial effect upon HGF-induced internalization of c-Met. However, it consistently reduced the rate of accumulation of c-Met in the perinuclear compartment (Fig. 2A). c-Met is consequently retracted in an early endosomal compartment at 120 min of stimulation (Fig. 2B, compare with Fig. 1F). Using the semi-automatic assessment of fluorescent vesicle distribution, we quantified this effect at 15 and 120 min of stimulation as shown in Table I. For example, at 15 min, BIM-I reduces the relative distance of c-Met from the cell boundary to nuclear boundary by 14% (p < 0.001). The BIM-I effect is specific to c-Met,
FIG. 1. HGF induces rapid c-Met-HGF complex internalization and traffic through an endosomal compartment. A and C–G, representative images corresponding to baso-medial Z-sections obtained by confocal microscopy. Bar, 20 μm. A, HeLa cells were stimulated with HGF for the indicated times between 0 and 480 min. They were then fixed and immunostained for c-Met (green). Nuclei (0–120 min only) were stained with propidium iodide (red). Arrows indicate examples of the localization of c-Met. B, time course of vesicle movement. Cells were stimulated with HGF for 5, 15, 30, or 120 min, fixed, and then processed for immunofluorescence for c-Met; nuclei were stained by propidium iodide. Fluorescence images of cell nuclei and vesicles were acquired with a CCD camera. The diagram summarizes the semi-automatic assessment of vesicle movement (see description of the technique under “Experimental Procedures”). C, HeLa cells were transfected by myc-AP180-C and stimulated 24 h later by HGF for 0 or 15 min. Cells were fixed and immunostained for myc (red) and c-Met (green). Arrows indicate examples of vesicular c-Met, which appears only in non-transfected cells. D, HeLa cells were pretreated with dansylcadaverine (200 μm), stimulated by HGF for 0 or 15 min, fixed, and immunostained for c-Met (green). Arrows indicate examples of the localization of c-Met. E, cells were stimulated with HGF for 0 or 60 min in the absence (control) or presence of MG132 (20 μM) or lactacystin (10 μM) added, respectively, 60 and 120 min before HGF. The experiments were performed such that, for times 0 and 120 min, the cells had been incubated in MG132 for a total of 120 min, or 180 min for lactacystin. Immunofluorescence was performed for c-Met (green) and nuclei (red) were stained with propidium iodide. Arrows indicate examples of vesicular c-Met. F, double immunostaining for c-Met (green) and EEA1 (red) after 0, 15, or 120 min of HGF stimulation. G, double immunostaining for c-Met (green) and HGF (red) after 0, 15, or 120 min of HGF stimulation. Regions of co-localization appear in yellow (arrows indicate examples).
because transferrin distribution is not modified after rhof treatment (Fig. 2C).

To confirm the effect of PKC on this trans-cytosolic traffic of c-Met the effect of the direct PKC activator TPA was assessed. In combination with HGF, TPA was found to promote the perinuclear accumulation of internalized c-Met (Fig. 2D). This effect was also blocked by BIM-I.

**The Trans-cytosolic Movement of c-Met Vesicles Is Microtubule-dependent**—The effects of PKC inhibition/activation indicate that trans-cytosolic traffic of c-Met is an active, regulated process. The basis of this active traffic was investigated in relation to the requirement for cytoskeletal integrity. Microtubules have been shown to be required for traffic of endocytosed receptors (see Refs. 24 and 25). Disruption of the microtubule

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**Table I**

| Treatment | Time | Relative distance, mean ± S.D. | Number of cells | p value (ANOVA) |
|-----------|------|-------------------------------|----------------|----------------|
| HGF       | 15   | 0.65 ± 0.04                   | 29             |                |
| HGF       | 120  | 0.76 ± 0.10                   | 31             | +a             |
| Bim       | 15   | 0.56 ± 0.07                   | 51             | ***            |
| Bim       | 120  | 0.68 ± 0.04                   | 24             | NS             |
| Vinb.     | 15   | 0.58 ± 0.06                   | 13             | NS             |
| Vinb.     | 120  | 0.57 ± 0.04                   | 74             | ***            |
| Taxol     | 15   | 0.51 ± 0.05                   | 71             | ***            |
| Taxol     | 120  | 0.59 ± 0.06                   | 58             | **             |

*a* + indicates a significant difference compared to HGF, 5 min.
network with vinblastine or its stabilization with taxol had no substantial effect upon HGF-induced internalization. However, after 240 min of HGF stimulation, only a limited colocalization was detected between c-Met and LBPA (data not shown) or LysoTracker (Fig. 4D) when compared with GM130, indicating that c-Met degradation is not substantially routed through the lysosomal pathway. In addition, co-localized immunofluorescence of LysoTracker is much less evident with c-Met than with EGFR after stimulation with HGF or EGF, respectively (Fig. 4E). It is also notable that perinuclear c-Met displays a distinct distribution compared with g-tubulin (Fig. 4F).

C-Met Degradation Is Proteasome-dependent and Independent of Its Post-endocytic Traffic—To distinguish proteasome and lysosomal directed c-Met degradation, proteasome inhibitors were employed. Both MG132 and lactacystin completely blocked p145c-Met degradation (Fig. 5A). By contrast, MG132 blocks only 24% of EGFR degradation after 120 min of EGF stimulation (Fig. 5B). Previous studies provided evidence that the primary effect of proteasome inhibition on c-Met degradation occurs indirectly through an effect on c-Met endocytosis (16). Although no effects of these agents on endocytosis had been observed (see above) the post-endocytic action of the inhibitors was confirmed by application 10 min after HGF, at a time when the bulk of c-Met was already internalized (see Fig. 1A). This post-treatment with MG132 also produced a complete block in HGF-induced p145c-Met degradation (Fig. 5C). In contrast to previous result (16), we do not detect any block of c-Met degradation in presence of the vacuolar-type H+ -ATPase inhibitor concanamycin A used at the same concentration (Fig. 5D).

Similar results have been obtained with bafilomycin A1 (data not shown). To determine whether the engagement of proteasome-mediated degradation was particular to the perinuclear compartment, delivery to this compartment was blocked by pretreating the cells with BIM-I, vinblastine, and taxol or, conversely, was promoted by TPA. None of these agents affected HGF-induced p145c-Met degradation (Fig. 5, E and F). Moreover, we found that proteasomal degradation can still be blocked in the presence of vinblastine (Fig. 5G). This indicates that engagement of the proteasome machinery is not restricted to the perinuclear compartment and that post-endocytic c-Met traffic and degradation are independent.

DISCUSSION

The endocytic and degradative pathway of c-Met has been detailed here for HeLa cells. The studies demonstrate that the bulk of internalized c-Met is delivered in a microtubule-dependent, PKC-controlled manner to a perinuclear compart-

![Image](72x430 to 292x737)
Fig. 4. The perinuclear c-Met corresponds in part to the Golgi. A, cells were stimulated by HGF for 0, 5, 10, 20, 30, 60, 120, 240, or 480 min, and Western blots for c-Met performed. A representative example of a Western blot is shown; p170 corresponding to the c-Met precursor and p145 to the β chain of c-Met. The graph represents the c-Met protein (p145 and p170) content as a function of the time of HGF stimulation. This was determined and was quantified by densitometric analysis of c-Met immunoblots (NIH Image 1.61/ppc), each value corresponding to the mean from four independent experiments. The results are indicated as percentage of protein increases or decreases as compared with time 0. *, p < 0.05; **, p < 0.01; ***, p < 0.005. B and C, HeLa cells were stimulated with HGF for 0, 60, 120, or 240 min in the absence (control) or in the presence of 50 μg/ml cycloheximide. The experiments were performed so that cells were incubated in cycloheximide for a total of 250 min. B, a representative Western blot for c-Met and α-tubulin at each time point is shown. C, representative confocal baso-medial sections at times 0 and 240 min showing double immunofluorescence for c-Met (green) and the Golgi marker p115 (red). Regions of co-localization appear in yellow or orange (see arrows for examples). Bar, 20 μm. D, representative confocal baso-medial sections at time 240 min of triple immunofluorescence for c-Met (green), Golgi marker GM130 (blue), and LysoTracker (red). Cells were incubated with LysoTracker (50 nM) 120 min before their fixation. Bar, 40 μm. E, representative confocal baso-medial sections of double immunofluorescence for LysoTracker (red) and c-Met or EGFR (green) after 120 min of HGF or EGF stimulation, respectively. Bar, 40 μm. F, representative confocal baso-medial sections at time 240 min of double immunofluorescence for c-Met (green) and γ-tubulin (red). Bar, 20 μm.
ment, from where it is subsequently degraded via a proteasome-dependent mechanism. Contrary to previous studies (16), we find no evidence for proteasome-sensitive endocytosis per se. On prolonged exposure to HGF, the c-Met p170 precursor becomes a significant form, and this correlates with the immunofluorescence data that indicates a Golgi localization. This late phase localization is consistent with the observation by Kamei and colleagues (26) of a perinuclear localization after 8 h of HGF treatment. Internalized c-Met is progressively degraded by a proteasomal pathway, and the lysosomal pathway does not play a substantial role. Although temporally related to its arrival, the degradation of p145-c-Met is not restricted to this perinuclear compartment, because inhibition of trans-cytosolic traffic (PKC inhibitors and microtubule disruption) or its promotion (PKC activation) does not alter the kinetics of degradation. Thus, susceptibility to proteasome degradation is not a direct consequence of traffic.

The mechanism of c-Met internalization appears to involve clathrin-mediated endocytosis, being inhibited by a pretreatment of cells with dansylcadaverine or by overexpression of the COOH part of AP180 (27) as a consequence of the blocking of clathrin-coated pit formation. The COOH domain of AP180 is able to bind clathrin and to stimulate cage assembly in vitro. These results are consistent with the finding that c-Met endocytosis is impaired by mutant dynamin expression (16). c-Met degradation is dependent on its internalization, because no HGF-dependent degradation is seen in the presence of dansylcadaverine (data not shown). Similarly, it has been shown
by others that expression of a dominant interfering mutant of endophilin (28) or mutant dynamin (16) impairs c-Met internalization and down-regulation.

In line with the studies by Jeffers et al. (13) we observed that HGF degradation of c-Met is under an absolute dependence of proteasome activity. By contrast, in HeLa cells under these conditions, the proteasomal pathway is responsible for only 24% of EGFR degradation. As noted above, we do not find that the proteasome/ubiquitination is implicated in c-Met internalization. This seems to be the case for the growth hormone receptor (17). As recently published, EGFR would not appear to be a direct proteasomal target but its lysosomal sorting is influenced by proteasome inhibition (29). Such indirect proteasome function on c-Met degradation is unlikely, because, contrary to the conclusion of Hammond et al. (16), we show that the perinuclear c-Met does not correspond to late endosomes/lysosomes and the co-localization with LysoTracker is substantially stronger for EGFR than c-Met. Consistent with this, no substantial effects of bafilomycin or concanamycin were observed on c-Met degradation; this is in contrast with the data of Hammond et al. (16) obtained with concanamycin. This implies that the alternative degradative pathway requiring lysosomal acidification is not a major one for c-Met. It cannot be excluded that a small part of the endocytosed c-Met is degraded by the lysosomal pathway. Indeed it is possible that a balance exists between proteasomal (dominating for c-Met) and lysosomal (dominating for EGFR) pathways that could be modified depending on cell type and/or different physiological or pathological conditions.

The analysis of the trans-cytosolic traffic of c-Met was facilitated by application of software that permits quantitation of the c-Met migration toward the nuclear boundary, where c-Met is destined within the first few hours. This provides clear statistical evidence on the process and the effects of inhibitors and activators. This was exploited to show for example that PKC stimulates c-Met trans-cytosolic traffic. PKC activity has been shown to increase or induce the internalization of the sst2A somatostatin receptor and the γ-aminobutyric acid type A receptor and to be absolutely required for parathyroid hormone receptor 1 internalization (18–20). By contrast, the activation of PKC decreased the rate of internalization of the cystic fibrosis membrane conductance regulator (30) and has been shown to inhibit the transfer of EGFR from early to late endosomes (31). In the present work, PKC inhibition or activation does not block c-Met internalization but impairs or promotes, respectively, the traffic from an early endosomal compartment to the perinuclear compartment. Kamei et al. (26) reported that long term (2–18 h) treatment with TPA alone induces c-Met internalization and its perinuclear accumulation. Here, acute treatment with TPA (25 min) did not modify c-Met plasma membrane expression. We also found that the BIM-I effect on traffic is still observed in cells pretreated with cycloheximide, indicating that we are not following modified movement of the newly synthesized c-Met.

It has been demonstrated for other receptors that transport from early to late endosomes can be microtubule-dependent. For example, perturbing cytoskeletal integrity by the use of microtubule depolymerizing agents such as nocodazole (32) or the microtubule-stabilizing drug taxol (25, 33), can inhibit EGFR or asialoglycoprotein receptor traffic. It is of note in this context that PKC has been reported to influence microtubule dynamics in a number of cell types (25, 33, 34). It is possible, that PKC might increase the turnover of attachment/detachment of c-Met vesicles to the microtubules or perhaps that PKC promotes an increase of microtubule lifetimes as has been shown recently in neuronal growth cones (35). It was noted that BIM-I by itself does not modify the integrity of the microtubule cytoskeleton (data not shown). Multiple aspects of PKC control of c-Met behavior may be relevant to HGF responses. Thus, it has been shown that PKC activity can be required for HGF-induced cell proliferation (36) and invasion (37). The extent to which this is dependent upon the intrinsic dynamics of c-Met traffic is as yet unclear, however, the precedent of PKC control of β1-integrin-dependent migration though the control of β1-integrin traffic (38, 39) suggests an important role for controlling c-Met traffic. On the other hand, PKC can play a negative role on c-Met function such as a decrease in HGF-induced cell tubulogenesis (40, 41).

There is evidence of a relationship between traffic and degradation for a variety of receptors. For instance, PKC reduces significantly EGFR degradation in parallel to the inhibition of EGFR trafficking to the late endosome (31). Another relevant example is that the modification of asialoglycoprotein trafficking by taxol is accomplished by an inhibition of its degradation (25). In the present study, PKC inhibitors, activators, and microtubule-disrupting agents do not modify the degradation of c-Met. These findings indicate that c-Met degradation does not require trafficking to a late endosomal compartment as has been proposed (16). This is consistent with the fact that c-Met does not extensively traffic through the late endosome pathway as discussed above. Consequently, it is concluded that the engagement of the proteasome machinery is not restricted to the perinuclear compartment. This is evidenced by the finding that the extent of degradation of c-Met remains unaffected and indeed can still be blocked by proteasome inhibition when the microtubule cytoskeleton is disrupted. This is also consistent with the finding that the mammalian proteasome is localized all over the cell (in contrast to yeast proteasomes, which mainly localize to the nuclear envelope/endoplasmic reticulum membrane network (42). Thus, susceptibility to proteasome degradation for c-Met is not a direct consequence of traffic.

The endocytosis of ligand-activated receptors has been considered to be the initiating step in its desensitization. Recent studies, however, have provided evidence that this removal from the plasma membrane may not of itself cause signal attenuation. In fact, there is growing evidence that receptors remain competent to signal in endosomal compartments and that the nature of the signal output is distinct in these compartments (43, 44). As we show here for c-Met, the rate of receptor degradation is much slower than the rate of internalization. Consequently, we detect the accumulation of pools of intracellular c-Met and HGF. It is likely that particular c-Met signals are generated within these distinct endosomal compartments. However, the demonstration here, that HGF-induced c-Met degradation can be effected equally from the various internalized compartments, indicates that this degradative pathway dominates those that control traffic (e.g. PKC) and endosomal signaling.

In summary, this study identifies a PKC-regulated step in microtubule-dependent traffic of c-Met. These properties are exploited to demonstrate that proteasome-dependent degradation of internalized c-Met is not a consequence of post-endocytic traffic.

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