Gastrointestinal Hormones Cause Rapid c-Met Receptor Down-regulation by a Novel Mechanism Involving Clathrin-mediated Endocytosis and a Lysosome-dependent Mechanism*

The activated c-Met receptor has potent effects on normal tissues and tumors. c-Met levels are regulated by hepatocyte growth factor (HGF); however, it is unknown if they can be regulated by gastrointestinal (GI) hormones. c-Met is found in many GI tissues/tumors that possess GI hormone receptors. We studied the effect of GI hormones on c-Met in rat pancreatic acini, which possess both receptors. CCK-8, carbachol, and bombesin, but not VIP/secretin, decreased c-Met. CCK-8 caused rapid and potent c-Met down-regulation and abolished HGF-induced c-Met and Gab1 tyrosine phosphorylation, while stimulating c-Met serine phosphorylation. The effect of cholecystokinin (CCK) was also seen in intact acini using immunofluorescence, in a biotinylated fraction representing membrane proteins, in single acinar cells, in Panc-1 tumor cells, and in vivo in rats injected with CCK. CCK-8 did not decrease cell viability or overall responsiveness. GF109203X, thapsigargin, or their combination partially reversed the effect of CCK-8. In contrast to HGF-induced c-Met down-regulation, the effect of CCK was decreased by a lysosome inhibitor (concanamycin) but not the proteasome inhibitor lactacystin. Inhibitors of clathrin-mediated endocytosis blocked the effect of CCK. HGF but not CCK-8 caused c-Met ubiquitination. These results show CCK and other GI hormones can cause rapid c-Met down-regulation, which occurs by a novel mechanism. These results could be important for c-Met regulation in normal as well as in neoplastic tissue in the GI tract.

Growth factor receptor tyrosine kinases are membrane-bound proteins that are involved in multiple growth-related intracellular signaling processes. After activation by their ligand, these receptors promote intracellular signals by phosphorylation of intracellular signaling proteins leading to the activation of intracellular signaling cascades (1, 2). Increasing interest is being placed on the regulation of growth factor receptor levels and their signaling because of the growing evidence that receptor tyrosine kinases play an important role in disease, including the development and growth of various malignancies (3). c-Met is a membrane-bound growth factor tyrosine kinase receptor, which initially was described as a cellular oncogene (4) and has been shown to be essential for normal organ, placenta, muscle, and central nervous development in the mouse model (5). The c-Met receptor is predominantly expressed in epithelial cells and is activated by its ligand, hepatocyte growth factor (HGF), which leads to the activation of signaling cascades promoting cellular growth, increased cell motility, invasive morphogenic programs, and anti-apoptosis (1, 2). The c-Met receptor is a 190-kDa membrane receptor protein that consists of an entirely extracellular 50-kDa α-chain and a transmembrane 145-kDa β-chain, whose intracellular domain has intrinsic tyrosine kinase activity that is essential for phosphorylating downstream signaling molecules (1, 2). Aberrant c-Met signaling has been shown to play an important role in a variety of malignancies, including numerous cancers of the gastrointestinal (GI) tract, such as pancreatic cancer (6–10). Understanding the regulatory mechanisms involved in c-Met kinase expression and signaling is important because it has been demonstrated for other malignancies that it is possible to pharmacologically influence aberrant tyrosine kinase signaling and therefore influence disease progression (3).

c-Met levels and signaling can be negatively regulated by binding of HGF to its receptor (11), as well by other stimuli (6, 12–14), including lysophosphatidic acid which is an activator of G-protein-coupled receptors (GPCRs) (15). In the GI tract many GI hormones/neurotransmitters mediate their cellular actions through GPCRs (16). These receptors occur widely in GI tissues and tumors and are frequently present along with...
CCK-stimulated c-Met Down-regulation

c-Met and other growth factor receptors (8, 9, 17–20). Although studies report that GI hormone/neurotransmitter receptors can actively regulate the expression and signaling of some growth factor receptors like the EGF receptor (21–23), it is unknown if GI hormones/neurotransmitters can influence the c-Met receptor. The pancreatic acinar cell model provides an excellent model to address this question because these cells express numerous functional GI hormone/neurotransmitter receptors as well as the c-Met receptor, which has been reported previously to be highly responsive to HGF in pancreatic acini, activating downstream signaling cascades that are linked to growth, anti-apoptosis, and increased cell motility (16, 18, 24). Activation of c-Met has been shown to have potent growth effects on all pancreatic tissues, including acini, duct cells, islet cells, as well as cancers derived from pancreatic tissues (20, 25–28). These pancreatic neoplasms frequently over-express c-Met, and studies show activation of c-Met is important in both the neoplastic progression and growth (20, 29–34). Furthermore, in pancreatic acinar cells, c-Met plays an important role both in their development as well as in their regenerative response to acute injury as occurs in acute and/or chronic pancreatitis (35). Therefore, to attempt to examine the possible influence of activated GI hormone/neurotransmitter receptors on c-Met receptor levels and signaling and the mechanisms involved, we used isolated pancreatic acini because of their responsiveness to these agents as well as the fact that HGF has potent effects on these cells in vivo in both physiological and pathological processes.

EXPERIMENTAL PROCEDURES

Materials

Male Sprague-Dawley rats (150–250 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health, Bethesda. Mouse anti-c-Met, rabbit anti-phospho-Gab1-pY627, rabbit anti-phospho-c-Met-pY1234/1235, rabbit anti-EGFR, anti-tubulin, and rabbit anti-phospho-EGFR-pY1068 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal anti-c-Met (B-2), rabbit polyclonal anti-c-Met (H-190), rabbit polyclonal anti-c-Met (C-12), rabbit polyclonal anti-c-Met (SP260), rabbit anti-phospho-c-Met-pS985, rabbit anti-PDGFR, mouse anti-ubiquitin, bovine anti-goat horseradish peroxidase conjugate, and anti-rabbit horseradish peroxidase conjugate antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-β-actin antibody was from Abcam, Inc. (Cambridge, MA). Anti-E-cadherin was purchased from BD Biosciences. Anti-calpain was from BIOSOURCE. Tris/HCl, pH 8.0 and 7.5, was from Mediatech, Inc. (Herndon, VA). 2-Mercaptoethanol, protein assay solution, and SDS were from Bio-Rad. CaCl₂ and MgCl₂ were from Quality Biological, Inc. (Gaithersburg, MD). Dulbecco’s PBS, glutamine (200 mM), Tris/glycine/SDS buffer (10×), and Tris/glycine buffer (10×) were from BIOSOURCE. Minimal essential media vitamin solution, basal medium Eagle’s amino acids 100×, and Tris/glycine gels were from Invitrogen. C-terminal octapeptide of cholecystokinin (CCK-8), hepatocyte growth factor (HGF), bombesin, vasoactive intestinal peptide (VIP), and secretin were from Bachem Bioscience Inc. (King of Prussia, PA). EGF, thapsigargin, GF109203X, A23187, concanamycin A, concanavalin A, phenylarsine oxide, and deoxycholic acid were from Calbiochem. Carbachol, dimethyl sulfoxide (Me₂SO), 12-O-tetradecanoylphorbol-13-acetate (TPA), 1-glutamic acid, fumaric acid, pyruvic acid, trypsin inhibitor, HEPES, Triton X, Tween®, Cellytic-M buffer, phenylmethylsulfonyl fluoride, EGTA, sucrose, sodium orthovanadate, and sodium azide were from Sigma. Albumin standard, protein G, SuperSignal West (Pico, Femto) chemiluminescent substrate, EZ-Link Sulfo NHS-S-S-biotin, immobilized avidin, and stripping buffer were from Pierce. Protease inhibitor tablets were from Roche Applied Science. Purified collagenase (type CLSPA) was from Worthington. Nitrocellulose membranes were from Schleicher & Schuell. Sterile saline solution was from Braun Medical (Barcelona, Spain). LIVE/DEAD viability/cytotoxicity kit and Alexa 488- and 555-conjugated anti-rabbit secondary antibody were from Molecular Probes (Eugene, OR). Poly-L-lysine-coated slides and sample chambers were from Wescor (Logan, UT). Polyclonal goat anti-murine HGF receptor antibody was from R & D Systems (Minneapolis, MN). Human CCKA receptor (cloned into pcDNA 3.1+) was from UMR cDNA resource center (Rolla, MO). Panc-1 cells were from ATCC (Manassas, VA).

Methods

Tissue Preparation—Pancreatic acini were obtained by collagenase digestion as described previously (36). Single pancreatic acinar cells were isolated as reported previously (37). Standard incubation solution contained 25.5 mM HEPES, pH 7.45, 98 mM NaCl, 6 mM KCl, 2.5 mM NaH₂PO₄, 5 mM sodium pyruvate, 5 mM sodium glutamate, 5 mM sodium fumarate, 11.5 mM glucose, 0.5 mM CaCl₂, 1 mM MgCl₂, 1 mM glutamine, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture, and 1% (v/v) amino acid mixture.

Acini Stimulation—After collagenase digestion, dispersed acini were preincubated in standard incubation solution for 2 h at 37 °C with or without inhibitors as described. After preincubation, 1-ml aliquots of dispersed acini were incubated at 37 °C with or without stimulants as described. Cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) deoxycholate, 0.1% (w/v) sodium azide, 1 mM EGTA, 0.4 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 protease inhibitor tablet per 10 ml). After sonication, lysates were centrifuged at 10,000 × g for 15 min at 4 °C, and protein concentration was measured using Bio-Rad protein assay reagent. Equal amounts of samples were analyzed by SDS-PAGE and Western blotting.

Analysis of Ubiquitinated c-Met Receptor—After protein measurement, equal amounts of lysates were incubated with 4 μg of mouse anti-c-Met (B-2) antibody (Santa Cruz Biotechnology) for 120 min at 4 °C and then for an additional 60 min with 25 μL of protein G-coupledagarose beads under constant agitation. After incubation, beads were washed twice in ice-cold lysis buffer, then twice in ice-cold phosphate-buffered saline, and analyzed by SDS-PAGE and Western blotting using mouse polyclonal anti-ubiquitin antibody.

Western Blotting—Western blotting was performed as described previously (36). Whole cell lysates, immunoprecipi-
tates, or lysates of subcellular fractions were subjected to SDS-PAGE using 4–20% Tris/glycine gels. After electrophoresis protein was transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer at room temperature, washed twice for 10 min in washing buffer, and treated with stripping buffer (Pierce) for 30 min at room temperature. A thin layer of cells was deposited onto poly-L-lysine-coated glass slides by cytoscraping (Cytopro cytcentrifuge, Wescor, Logan, UT) at 1000 rpm for 5 min. Slides were air-dried for 24 h, blocked in blocking buffer (10% isotype-matched serum, 1% bovine serum albumin in PBS, pH 7.5) for 60 min, and incubated with a polyclonal anti-c-Met antibody (1:50; R & D Systems), a polyclonal anti-phospho-c-Met (Y1234/1235; Cell Signaling) antibody at a dilution of 1:100, or a combination of a polyclonal anti-c-Met antibody (1:50; R & D Systems) and an anti-E-cadherin antibody (1:100; Cell Signaling) overnight at 4 °C. Reactivity was demonstrated by incubation with an Alexa 488-conjugated rabbit anti-goat and Alexa 555-conjugated rabbit anti-mouse or Alexa 488-conjugated goat anti-rabbit secondary antibodies (1:500, Molecular Probes) for 1 h at room temperature. Control samples were prepared by replacing the primary antibody with an isotype-matched serum. Fluorescent images were collected using a Leica CTR5000 microscope with a ×63 objective in oil immersion. Multiple images using different focal points at 0.1 μm steps to cover the entire thickness of the sample (z-stacking) were acquired using a RetigaExi camera (Qimaging, Burnaby, British Columbia, Canada) and Volocity software (Improvision, Lexington, MA). All images of one experiment were acquired using the same settings. Post-image collection restoration was executed using the software’s iterative restoration tool. Images were cropped in Adobe Photoshop CS with the same settings being applied to each image of one experiment, and the layout of the final figure was finished in Adobe Illustrator. To analyze c-Met staining intensity, 10 representative cytoplasmic regions of interest of the same size were selected in Volocity, and the mean signal intensity was determined using the measurement tool (this determination is independent from picture settings like contrast or levels). To determine co-localization of c-Met and E-cadherin at the membrane, 10 representative regions of interest of comparable size were selected in the membrane region, and co-localization was determined using the co-localization tool and calculating the co-localization coefficient.

**Cell Viability Experiments**—The viability of pancreatic acinar cells was determined using the LIVE/DEAD viability/cytotoxicity kit for animal cells from Molecular Probes following the directions provided by the manufacturer (Molecular Probes, Eugene, OR). Briefly, pancreatic acini were incubated with 100 nM CCK for the time indicated, washed twice with PBS at 37 °C, and placed on 22-mm square coverslips, pretreated overnight with poly-L-lysine, and allowed to dry. Cells were then covered with 100–150 μl of LIVE/DEAD assay reagents (1 μM ethidium homodimer-1 and 1 μM calcein-AM) and incubated for 30 min at room temperature. After washing with PBS, samples were examined with an inverted fluorescence microscope (Nikon Diaphot 200; Nikon, Tokyo, Japan). Two images were taken under optimal excitation/emission wavelengths for each dye (calcein = 494/517 nm; ethidium homodimer-1 (on DNA) = 528/617 nm) from different regions of the slide randomly chosen. Both images were merged using Confocal Assistant, version 4.02 (Bio-Rad), and the percentage of dead and live cells was objectively quantified on the collected images using Scion Image for Windows, version 4.02 (Scion Corp., Frederick, MD).

**In Vivo Experiments**—Adult male Wistar rats (200 and 225 g) were fed a regular rodent diet (Panlab, Barcelona, Spain) and were divided into four groups of four rats each. The first group was injected intraperitoneally with sterile saline solution at 37 °C and sacrificed 20 min after the injection. The second group was injected intraperitoneally with one dose of CCK-8 (10 μg/kg, prepared in sterile saline solution at 37 °C as excipient) and sacrificed 20 min after the injection. The third group was injected intraperitoneally with two sequential doses of saline 20 min apart, and sacrificed 20 min after the last inject-
TABLE 1  
Characteristics of c-Met receptor protein level down-regulation by GI hormones/neurotransmitters and ability of inhibitors of intracellular signaling pathways, protein degradation, and clathrin-mediated endocytosis to reverse CCK-8 effects on c-Met level in rat pancreatic acini

| Treatment                              | c-Met levels after GI hormone/neurotransmitter treatment (% of control)* | IC_{50} (nM) | α-Actin antibody |
|----------------------------------------|-------------------------------------------------|---------------|-----------------|
| Control                                | 100 ± 3                                         | 0             | No effect       |
| CCK-8 (100 nM)                         | 15 ± 5                                          | 6.7           | Complete inhibition of CCK effect |
| CCK-JMV (1 μM)                         | 105 ± 7                                         | 7             | Complete inhibition of CCK effect |
| Carbachol (100 μM)                     | 68 ± 9                                          | 3             | Complete inhibition of CCK effect |
| Bombesin (1 μM)                        | 74 ± 10                                         | 3             | Complete inhibition of CCK effect |
| Secretin (1 μM)                        | 108 ± 9                                         | 3             | Complete inhibition of CCK effect |
| VIP (100 nM)                           | 108 ± 5                                         | 3             | Complete inhibition of CCK effect |

* Incubation time was 45 min. Results were calculated from the data of 5–7 independent experiments using band density measurement (Kodak ID image analysis; representative Western blots shown in Figs. 1 and 8). Values are expressed as percent of control c-Met receptor protein levels ± S.E. (i.e., no treatment).

† p < 0.0001 for CCK and p < 0.05 for carbachol and bombesin compared with control c-Met levels.

‡ p < 0.001, p < 0.05, and p < 0.001 for GF109203X, thapsigargin, and GF109203 + thapsigargin compared with C-Met levels after CCK stimulation, respectively.

§ Time course results were calculated from four independent experiments (Fig. 3). Values are expressed in minutes ± S.E. IC_{50} value was calculated from four independent experiments (Fig. 4). Value is expressed in ms ± S.E.

** p < 0.01 for concanamycin A is compared with CCK alone.

A complete inhibition of CCK effect was observed for all treatments except for carbachol (100 μM), bombesin (1 μM), and VIP (100 nM).

RESULTS

Effect of GI Hormones/Neurotransmitters on c-Met Protein Levels in Rat Pancreatic Acini—To explore whether GI hormones/neurotransmitters can alter c-Met protein levels, we studied their effects in rat pancreatic acini, which are reported to possess receptors for c-Met as well as numerous GI hormone/neurotransmitters (16, 38). Pancreatic acini were stimulated by various GI hormones/neurotransmitters at concentrations known to activate pancreatic acinar cells (38, 39), and c-Met protein levels were analyzed using Western blotting (Fig. 1). CCK-8, carbachol, and bombesin, which activate PKC and increase intracellular calcium, but not secretin or VIP, which activate protein kinase A (16), caused down-regulation of c-Met levels (Fig. 1). Specifically, stimulating pancreatic acini with CCK-8 (100 nM), carbachol (100 μM), or bombesin (1 μM) for 45 min (Fig. 1, lanes 2, 4, and 5) resulted in an 85% (p < 0.0001), 32% (p < 0.05), and 26% (p < 0.05) decrease of c-Met levels compared with control, respectively (Fig. 1 and Table 1). CCK-JMV (1 μM), which is an antagonist of the low affinity state and agonist of the high affinity state of the CCK receptor (40), had no effect on c-Met receptor levels (Fig. 1, lane 3). These results demonstrate for the first time that c-Met protein levels can be negatively regulated by activation of some GI hormone/neurotransmitter GPCRs.

Effect of CCK-8 Is Specific for the c-Met Receptor and Does Not Affect PDGF or EGF Receptor Levels—Because CCK is a physiological regulator of pancreatic secretion (41) and CCK-8 had the greatest effect on c-Met levels (Fig. 1), its effect was studied in detail. Similar effects on c-Met protein levels occurred after 45 min of 100 nM CCK-8 treatment when analyzing pancreatic acini by Western blotting with four different antibodies mapping for different regions of the cytoplasmic part of the trans-
because of an indirect effect (the c-Met down-regulation observed in pancreatic acini is not due to the tumor cell line Panc-1 stably transfected with the CCKA receptor or single acinar cells with or without CCK-8 (100 nM) for 45 min were subjected to SDS-PAGE and transferred to nitrocellulose membranes as described under “Experimental Procedures.” Panel A, membranes were analyzed by WB using mouse monoclonal anti-c-Met antibody (B-2; Santa Cruz Biotechnology). Panel B, membranes were analyzed by WB using rabbit polyclonal anti-c-Met antibody (C-12; Santa Cruz Biotechnology). Panel C, membranes were analyzed by WB using rabbit polyclonal anti-c-Met antibody (H-190; Santa Cruz Biotechnology). Panel D, membranes were analyzed by WB using rabbit polyclonal anti-PDGF antibody. Panel E, membranes were analyzed by WB using rabbit polyclonal anti-EGFR antibody. Panel F, to ensure equal loading of protein, membranes were re-probed using goat polyclonal anti-β-actin antibody. Bands were visualized by using chemiluminescence. Results are representative of data from at least four independent experiments.

membranous β-domain of the receptor (Fig. 2, panels A and B; SP260 from Santa Cruz Biotechnology, C-terminal data not shown; Cellular Signaling, 25H2, C-terminal data not shown) and one antibody mapping for the extracellular part of the β-domain of the receptor (Fig. 2, panel C). In contrast CCK-8 treatment had no effect on either the protein levels of the PDGF or the EGF receptor (Fig. 2, panels D and E). These results demonstrate that the effect of CCK-8 is not because of a general effect on all growth factor receptors and is unlikely to be due to a toxic effect on acini because it is selective for the c-Met receptor.

CCK-8 Causes c-Met Down-regulation in Panc-1 Cells and in Single Pancreatic Acinar Cells—We next wanted to verify that the c-Met down-regulation observed in pancreatic acini is not because of an indirect effect (e.g. mediator produced after CCK-8 stimulation by other cells contaminating the acini preparation) but by direct action of CCK-8 on acinar cells. Therefore, we analyzed the effect of CCK-8 on the human pancreatic tumor cell line Panc-1 stably transfected with the CCKA receptor. CCK-8 (100 nM, 60 min) caused a decrease in c-Met expression comparable with that seen in pancreatic acini (Fig. 3, upper panel). Furthermore, single acinar cells were isolated from rat pancreata. These cells undergo a more thorough purification than the dispersed acini preparation, making contamination by nonacinar cells improbable. In these cells, CCK-8 (100 nM, 60 min) caused a c-Met down-regulation comparable with that seen in dispersed acini (Fig. 3, lower panel). In both cells the CCK was not causing a general decrease in cellular protein because their was no change in tubulin with CCK-8 treatment (Fig. 3).

CCK-8 Causes Degradation of Cell Surface c-Met Receptors—To analyze the effect of CCK-8 on the functionally relevant pool of c-Met displayed at the cell surface, a surface biotinylation assay was conducted as described previously (42). In the total cell lysates at 45 min post-CCK-8 (100 nM), tubulin was unchanged; however, c-Met was markedly reduced (Fig. 4, top panel). In the biotinylated fraction representing cell surface proteins, CCK-8 treatment (45 min, 100 nM) led to a dramatic decrease of c-Met receptor, whereas the EGF receptor levels were not changed (Fig. 4, middle panel). This was further confirmed by a highly significant (p < 0.0001) decrease of the c-Met/EGFR ratio in the biotinylated fraction after CCK-8 treatment (Fig. 4, lower panel), demonstrating CCK-8 induces a selective degradation of c-Met located at the cell membrane. Control lysates of nonbiotinylated acini did not yield a specific signal for c-Met or EGFR after immunoprecipitation with immobilized avidin, confirming the specificity of our assay (data not shown).

CCK-8 Time Dependence and Dose-response Effect on c-Met Protein Levels—Down-regulation of c-Met receptor started after 10 min of 100 nM CCK-8 treatment and reached a maximum at 45 min (Fig. 5). No significant further decrease in protein levels occurred after 60 min of CCK-8 treatment (Fig. 5). The t1/2 calculated from data from four independent experiments was 20.1 ± 2.4 min, and the calculated time to maximum effect was 48.8 ± 3.8 min (Table 1). CCK-8 dose-dependently decreased c-Met protein levels (Fig. 6). The effect of CCK-8 was detectable at 1 nM after 45 min of incubation and reached a
CCK-stimulated c-Met Down-regulation

Maximum at 30 nM (Fig. 6). Further increasing the CCK concentration did not significantly decrease c-Met levels. The IC\textsubscript{50} values of CCK calculated from four independent experiments was 4.8 ± 0.4 nM (Table 1).

CCK-8 Induced Down-regulation in Situ—Because c-Met down-regulation was detectable by analyzing whole cell lysates using Western blotting, we postulated that a decrease in c-Met levels after CCK-8 treatment should also be detected using in situ immunofluorescence cytochemistry in intact rat pancreatic acini. Staining of untreated rat pancreatic acini for c-Met with the polyclonal goat anti-murine c-Met antibody from R & D Systems resulted in a predominantly membranous staining pattern (Fig. 7, panel A). After 45 min of 100 nM CCK-8 treatment, there was a loss of the membranous staining pattern and the overall cellular staining for c-Met markedly decreased (Fig. 7, panel B). These results demonstrate that CCK-8-induced c-Met down-regulation can be observed using both Western blotting and in situ immunocytochemistry.

CCK-8 Treatment Abolishes HGF-stimulated Phosphorylation of the c-Met Receptor and of the Downstream Signaling Molecule Gab1—Activation of the c-Met receptor by HGF leads to the activation of its tyrosine kinase and to autophosphorylation of the receptor, which results in the phosphorylation and activation of numerous downstream signaling pathways that lead to the growth and mitogenic and antiapoptotic effects promoted by HGF in epithelial cells (1, 2, 9, 18, 24). To assess the effect of CCK-induced down-regulation of c-Met on the ability of HGF to activate various cellular signaling cascades, we studied the effect of CCK alone and of CCK preincubation on HGF-induced phosphorylation of tyrosines 1234/1235 of c-Met and of tyrosine 627 of Gab1 (2, 43). Incubating pancreatic acini with CCK-8 alone did not affect tyrosine phosphorylation of Tyr-1234/1235 of c-Met or of Gab1 (data not shown). However, preincubating pancreatic acini with CCK-8 (100 nM) decreased HGF-induced tyrosine phosphoryl-

**FIGURE 4.** Surface biotinylation assay. Pancreatic acini were incubated with or without CCK (100 nM) for 45 min. After incubation, cells were biotinylated at 4 °C for 45 min and lysed. Total cell lysates were subjected to SDS-PAGE and analyzed by WB. Top, representative blot from four independent experiments. Top upper panel, membranes were analyzed using mouse monoclonal anti-c-Met antibody. Top lower panel, to ensure equal loading of protein, membranes were re-probed with rabbit polyclonal anti-tubulin antibody. Middle, to isolate biotinylated cell surface proteins, total cell lysates were submitted to immunoprecipitation with immobilized avidin beads. These beads were washed extensively, mixed with SDS sample buffer, and submitted to SDS-PAGE and WB. Representative blot is from four independent experiments. Middle upper panel, membranes were analyzed using mouse monoclonal anti-c-Met antibody. Middle lower panel, to verify that CCK-8 treatment leads to degradation of c-Met but not other growth factor receptors, membranes were re-probed with rabbit polyclonal anti-EGFR antibody. Bottom, the ratio c-Met/EGFR was calculated in the biotinylated fraction (lower panel). Data from four independent experiments are shown.

**FIGURE 5.** Time course of CCK-8 induced down-regulation of c-Met protein levels in rat pancreatic acini. Pancreatic acini were incubated with or without CCK-8 (100 nM) for the times indicated. After the incubations, cells were lysed, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by WB. Top, representative blot from four independent experiments. Top upper panel, membranes were analyzed using mouse monoclonal anti-c-Met antibody. Top lower panel, to ensure equal loading of protein, membranes were re-probed with goat polyclonal anti-β-actin antibody. Bands were visualized using chemiluminescence. Bottom, the graph shows data from band intensity measurements (mean ± S.E.) from four independent experiments. Values are expressed as percentage of control c-Met levels.
acini using immunocytochemistry (Fig. 9, panels A–C). Using an antibody specific for detecting phosphorylated tyrosine 1234/1235 of c-Met, incubation of pancreatic acini with HGF (1 nM) for 5 min without any CCK pretreatment resulted in a membranous staining pattern (Fig. 9, panel B). After 45 min of CCK-8 pretreatment, HGF-induced membranous staining was completely abolished (Fig. 9, panel C). Phosphorylation of serine 985 of c-Met after stimulation with HGF, TPA, increases in intracellular calcium or oxidative stress in other tissues is reported to result in inactivation of the c-Met kinase (7). HGF (1 nM) alone increased phosphorylation of serine 985 of c-Met (Fig. 8, 3rd panel, lane 2), and CCK-8 pretreatment increased this effect, and serine phosphorylation levels returned to base line after 30 min despite HGF stimulation (Fig. 8, 3rd panel). We found an increase in serine 985 phosphorylation levels caused by CCK-8 stimulation alone that returned to base line at 30 min (data not shown). These results demonstrate that CCK-8 decreases c-Met protein levels but also inhibits the ability of HGF to cause activation of the downstream signaling pathways of c-Met receptor. Furthermore, CCK-8 increases phosphorylation levels of serine 985 of the c-Met receptor.
CCK-stimulated c-Met Down-regulation

![Diagram showing CCK-stimulated c-Met Down-regulation](image)

**FIGURE 10. Responsiveness to EGF treatment and viability of rat pancreatic acini after CCK-8 incubation.** Top, after preincubation with or without CCK-8 (100 nM) for 45 min, pancreatic acini were incubated with or without EGF (10 nM). After the incubations, cells were lysed, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by WB. Representative blot of five independent experiments is shown. Top upper panel, membranes were analyzed using rabbit polyclonal anti-phospho-EGFR (Y1068) antibody. Top lower panel, membranes were analyzed using rabbit polyclonal anti-EGFR antibody. Bottom, pancreatic acini were incubated with or without CCK-8 (100 nM) for the times indicated. After incubation the viability of pancreatic acinar cells was determined using the LIVE/DEAD viability/cytotoxicity kit for animal cells as described under “Experimental Procedures.” The percentage of cells alive or dead was quantified using Scion Image software. The graph shows data from at least six independent experiments (mean ± S.E.). Values are expressed as percent of cells alive.

which in other cell systems has been linked to c-Met tyrosine kinase inactivation.

Pancreatic Acini Remain Viable, and EGF-induced Tyrosine Phosphorylation of the EGF Receptor Is Not Affected after CCK-8 Pretreatment—To further investigate whether the effect of CCK-8 on HGF-induced tyrosine phosphorylation could be explained by a general unresponsiveness of pancreatic acini either because of a potential toxic effect of CCK-8 at the concentrations used or because of a general inability of acini to respond to extracellular stimuli after CCK-8 treatment, the viability of acini and responsiveness of acini to other stimuli were examined after CCK-8 treatment (Fig. 10). Viability of pancreatic acini as determined by ethidium and calcein staining did not differ with or without CCK-8 treatment at a concentration of 100 nM after a 20-min (89 ± 2.6% versus 90 ± 2.0%), 60-min (91 ± 1.4% versus 87 ± 3.0%), or 90-min preincubation (86 ± 4.6% versus 87 ± 4.6%) (Fig. 10, bottom). Studies have shown that pancreatic acini possess EGF receptors and that their stimulation leads to the activation of various cellular signaling cascades (16, 18, 44). To investigate if pancreatic acini remain responsive to EGF treatment after CCK-8 pretreatment, we studied the ability of EGF to stimulate tyrosine phosphorylation of tyrosine 1068 of the EGF receptor with or without CCK-8 pretreatment (Fig. 10, top). Incubating pancreatic acini with EGF (10 nM) and or without pretreatment with 100 nM of CCK-8 for 45 min, a treatment that caused a complete loss of HGF-induced c-Met tyrosine phosphorylation (tyrosine 1234/1235, Fig. 8), resulted in similar increases in tyrosine phosphorylation levels of tyrosine 1068 of the EGF receptor (Fig. 10, top). These results demonstrate that pancreatic acini remain viable and responsive to extracellular stimuli after CCK-8 treatment and show that CCK-8 pretreatment is not having a general toxic effect on the acini.

Effect of PKC Inhibition and/or Depletion of Intracellular Calcium Stores on CCK-8-induced Down-regulation of the c-Met Receptor—CCK-8 activates its corresponding GPCR receptor, CCKA-R, which leads to activation of phospholipase C (PLC). PLC activation results in increases of intracellular diacylglycerol and inositol 1,4,5-trisphosphate, which in turn cause activation of PKC and an increase in intracellular calcium leading to the activation of downstream signaling mechanisms (16, 45). To determine directly whether CCK-8 activation of PKCs and/or alteration in intracellular calcium concentrations could be contributing to the down-regulation of c-Met, we examined the effect of the PKC inhibitor GF109203X alone or in combination with thapsigargin in a calcium-free medium, under conditions that deplete calcium from calcium stores in pancreatic acini (46). CCK-8-induced down-regulation of c-Met was partially inhibited by GF109203X (19 ± 3%) and thapsigargin (33 ± 7%) alone (Fig. 11, lanes 4 and 6; p < 0.01 and <0.05, respectively; see Table 1). Preincubating with a combination of both inhibitors resulted in 61 ± 6% inhibition of CCK-8 induced c-Met down-regulation (Fig. 11, lane 8; p < 0.001; see Table 1). These results show that the effect of CCK-8 on c-Met receptor protein levels is mediated partially through both activation of PKC and increases of intracellular calcium levels.

Inhibition of Clathrin-mediated Endocytosis Prevents c-Met Down-regulation by CCK-8—Down-regulation of membranous receptors by an active cellular mechanism frequently involves clathrin-mediated endocytosis, and c-Met has been reported to be internalized by clathrin-mediated mechanisms following HGF stimulation in HeLa cells (11, 47, 48). Therefore, we next investigated whether CCK-8-induced c-Met down-regulation is sensitive to inhibitors of clathrin-mediated endocytosis in rat pancreatic acini. Preincubating acini with various inhibitors of clathrin-mediated endocytosis (0.5 M sucrose, 20 μM phenylarsine oxide, or 0.25 mg/ml concanavalin A (49–52)) effectively prevented CCK-8-induced down-regulation of the c-Met receptor (Fig. 12, lanes 3–8; Table 1). These results demonstrate that CCK-8-induced c-Met down-regulation requires active endocytosis of the c-Met receptor through a clathrin-mediated endocytotic pathway.

CCK-8 Stimulates c-Met Internalization in Situ—As we found that CCK-8 stimulated c-Met receptor degradation through endocytosis, we hypothesized that, shortly after CCK-8 stimulation, an increase in intracellular c-Met should be detectable in intact acini. In nonstimulated pancreatic acini, the polyclonal anti-murine c-Met antibody (R & D Systems) used for this experiment demonstrated a predominantly membra-
CCK-stimulated c-Met Down-regulation

**CCK-stimulated c-Met Down-regulation**

Involving Both the Proteasomal and Lysosomal Pathway—Studies have shown in other tissues that HGF stimulates down-regulation of the c-Met receptor, a process that is sensitive to inhibition of the proteasome by lactacystin and to lysosome inhibition by concanamycin A (11). HGF-induced c-Met down-regulation has not been demonstrated in pancreatic acini. We found that in pancreatic acini HGF (1 nM) caused c-Met down-regulation beginning after 3 h of incubation (Fig. 14, lane 2). After 4 h of incubation c-Met levels decreased to 55 ± 2% of control levels (p < 0.0001). We next preincubated acini with proteasome and lysosome inhibitors under conditions shown previously to cause inhibition of these cascades (21). Preincubating acini with the proteasome inhibitor lactacystin (30 μM) markedly inhibited HGF-induced c-Met down-regulation (Fig. 14, lanes 4–6; p = 0.0014), whereas the lysosome inhibitor concanamycin A (100 nM) only partially reversed the effect of HGF (Fig. 14, lanes 7–9; p < 0.05). The partial inhibition by concanamycin A was not likely because of an ineffective use of the inhibitor at blocking lysosomal action in these cells, because when used with the same experimental conditions, it effectively blocked the effect of CCK on c-Met (Fig. 14, lane 6).

**CCK-8-induced c-Met Down-regulation Differs from That Induced by HGF in Its Sensitivity to Proteasome and Lysosome Inhibitors and in Its Ability to Cause c-Met Ubiquitination.—To determine whether CCK-8-induced c-Met down-regulation might follow a similar mechanism to that used by HGF, we next investigated the effects of the proteasome inhibitor lactacystin and of the lysosome inhibitor concanamycin A on CCK-8-induced c-Met down-regulation (Fig. 15, middle panel; see Table 1). In contrast to what was seen with HGF (Fig. 14, lane 6), preincubating acini with lactacystin (30 μM) had no effect on CCK-8-induced c-Met down-regulation (Fig. 15, top panel, lane 4). This was not likely because of the ineffective use of the lactacystin to block proteasome action in these cells, because when lactacystin was used under the same conditions, it effectively blocked the effect of CCK on c-Met (Fig. 14, lane 6). Also in contrast to what was seen with HGF (Fig. 14, lane 9), incubation with concanamycin A (100 nM) before CCK-8 stimulation caused c-Met levels to return to 71 ± 7% S.E. of control levels (Fig. 15, panel A, lane 6; p < 0.01). Studies have shown that HGF causes c-Met polyubiquitination, a process that is necessary for endosomal sorting of the c-Met receptor, which leads to its degradation in late lysosomes (11, 53–55). We therefore investigated whether HGF and/or CCK-8 cause ubiquitination of the c-Met receptor in rat pancreatic acini. We incubated pancreatic acini with either no additions, HGF or CCK-8, immunoprecipitated the c-Met receptor from cell lysates, and analyzed immunoprecipitates by Western blotting using antibody for ubiquitin (Fig. 15, panel B). Similar to reports in other publications, we found a massive increase in intracellular c-Met staining (Fig. 13, compare panels B and E). This was further verified by measuring intracellular intensity of the corresponding color channel in 10 representative regions of interest, which showed a significant increase after 20 min of CCK treatment (1563 ± 19 versus 2419 ± 81, p < 0.0001). Second, there was a loss of the membranous staining pattern, which was further validated by measuring co-localization of c-Met with the membrane protein E-cadherin in 10 representative regions of interest. There was a significant reduction in co-localization after 20 min of CCK-8 treatment (co-localization coefficient 0.80 ± 0.026 versus 0.62 ± 0.026, p < 0.01).
CCK-stimulated c-Met Down-regulation

FIGURE 13. CCK-8 stimulates internalization of c-Met in rat pancreatic acini. Pancreatic acini were incubated with no addition or CCK (100 nM) for a shorter period of time (20 min) as indicated. After stimulation cells were washed, fixed, and transferred onto poly-1-lysine-coated glass slides by cytocentrifugation as described under “Experimental Procedures.” Cells were then labeled using polyclonal goat anti-c-Met (R & D Systems) and polyclonal mouse anti-E-cadherin primary antibodies. Specific binding was detected using Alexa 488- and Alexa 555-conjugated secondary antibodies. The upper panel shows acini without the addition of primary antibody as a negative control. Fluorescent images were collected using a Leica CTR5000 microscope. Green staining represents staining for total c-Met; red represents staining for E-cadherin, and the nuclei were counterstained in blue with 4,6-diamidino-2-phenylindole. Shown are results of a typical experiment which is representative of three independent experiments.

CCK-8 treatment (Fig. 16, lane 3; \( p = 0.0011 \)). These results indicate that CCK-8 not only induces c-Met down-regulation in vitro but can also affect c-Met levels in vivo.

**DISCUSSION**

c-Met is a proto-oncogene that in normal cells functions as a membrane-bound receptor tyrosine kinase that is activated by its ligand, HGF. It has been shown for the c-Met receptor as well as for other growth factor receptors that receptor expression is regulated by cellular mechanisms and that binding of growth receptor ligand can trigger the down-regulation of membrane-bound receptor (47, 48). This effect is thought to be important to attenuate/terminate signaling from activated tyrosine kinase receptors to prevent cell overgrowth, a hypothesis that is supported by the fact that the disruption of the down-regulation mechanisms of the c-Met receptor was demonstrated to have malignant transforming effects on epithelial cells (58, 59). Understanding the mechanisms of c-Met receptor down-regulation is important because it has been shown that by actively down-regulating c-Met receptor levels, tumor growth and metastasis can be inhibited in epithelial tumors (17, 60, 61).

Physiologically, c-Met levels cannot only be regulated by its ligand HGF but also by activation of other receptor systems, such as the Notch receptor, the interferon-\( \alpha \) receptor, and the transforming growth factor-\( \beta 1 \) receptor (6, 12) or by signaling cascades triggered by oxidative stress (14), but it is not known if GPCR receptors of GI hormones/neurotransmitters can alter c-Met levels. In this study in order to explore the possibility that GI hormones/neurotransmitters can influence c-Met levels and to investigate the mechanisms involved, we studied their effect on c-Met levels as well as their effect on c-Met signaling in rat pancreatic acinar cells, which are known to express the c-Met receptor as well as numerous GI hormone/neurotransmitter receptors and are known to be highly responsive to a number of the most important GI hormones/neurotransmitters (16). To exclude that our observation could be due to an indirect effect on acini, e.g. mediated by cells possibly contaminating the acini preparation, and to establish the relevance in pancreatic cancer cells, we studied the effect of CCK-8 on c-Met expression in Panc-1 cells as well as single pancreatic acinar cells.

A number of our results support the conclusion that some but not other GI hormones/neurotransmitters can specifically down-regulate c-Met receptor protein levels in pancreatic acinar cells and in Panc-1 pancreatic cancer cells. First, CCK, car-
CCK-stimulated c-Met Down-regulation

**FIGURE 14. Effect of proteasome or lysosome inhibitors on HGF-induced c-Met receptor down-regulation.** Pancreatic acini were preincubated with no additions (○), lactacystin (30 μM) (■) for 2 h, or concanamycin A (100 nM) (▲) for 30 min and then incubated with or without HGF (1 nM) for the times indicated. The incubations, cells were lysed, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by WB. Top, representative blot from one of nine independent experiments is shown. Top upper panel, membranes were analyzed using mouse monoclonal anti-c-Met antibody. Top lower panel, to ensure equal loading of protein, membranes were re-probed with goat polyclonal anti-β-actin antibody. Bands were visualized using chemiluminescence. Representative blots from nine independent experiments are shown. Bottom, the graph shows data from band intensity measurements (mean ± S.E.) from nine independent experiments. Values are expressed as percentage of control c-Met levels. *, p = 0.0014; **, p < 0.05.

**FIGURE 15. Effect of proteasome or lysosome inhibitors on CCK-induced c-Met receptor down-regulation.** Top panel, pancreatic acini were preincubated with no addition, lactacystin (30 μM) for 2 h, or concanamycin A (100 nM) for 30 min and then incubated with or without CCK-8 (100 nM) for 45 min. After the incubations, cells were lysed, subjected to SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by WB. Top of upper panel, representative blot from one of five independent experiments is shown. Membranes were analyzed using rabbit polyclonal anti-c-Met antibody (H-190). To ensure equal loading of protein, membranes were re-probed with goat polyclonal anti-β-actin antibody. Bands were visualized using chemiluminescence. Representative blots from five independent experiments are shown. Bottom of upper panel, the graph shows data from band intensity measurements (mean ± S.E.) from five independent experiments. Values are expressed as percentage of control c-Met levels. *, p = 0.0001; **, p < 0.01.

Bachol and bombesin caused significant down-regulation of c-Met protein levels assessed by Western blotting, whereas secretin and vasoactive intestinal peptide had no effect. Rat pancreatic acinar cells, which were used in this study, possess specific receptors for each of these GI hormones/neurotransmitters, which at the concentrations used have been shown to cause marked changes in cell signaling and acinar cell function (16, 18, 45, 62, 63); therefore, the failure to see responses to vasoactive intestinal peptide and secretin was not because of a general unresponsiveness to these agents. These results support the conclusion that only selective GI hormones/neurotransmitters affect c-Met levels. Second, the effect of CCK on c-Met protein levels could also be seen in situ in intact pancreatic acini using immunocytochemistry. The effect of CCK on decreasing c-Met protein levels could be demonstrated in vivo in rats injected intraperitoneally with CCK. Third, the ability of CCK to down-regulate c-Met was specific for the c-Met receptor and not because of a general effect on growth factor receptors or cellular proteins, because we found that protein levels of the EGF receptor or PDGF receptor were not affected by treatment with CCK. Moreover, CCK caused degradation of biotinylated c-Met, but not EGFR at the plasma membrane, nor was the cellular level of tubulin changed. Furthermore, our results demonstrate that the effect on c-Met was not caused by a toxic effect of GI hormones/neurotransmitters on pancreatic acini. This was supported by our findings that CCK incubations of up to 90 min did not alter cell viability, that the effect of CCK was dose-dependent and was also observed at lower CCK concentrations, and that after CCK treatment EGFR stimulation resulted in a similar increase in the phosphorylation levels of tyrosine 1068 of the EGF receptor compared with control cells, demonstrating that the cells retained their overall responsiveness to other growth factors. Finally, the demonstration that CCK caused similar effects to dispersed acini in single enriched...
acinar cells and Panc1 cancer cells expressing CCKα receptors supports the conclusion that it is because of direct action mediated through the CCK receptors on these cells.

Because CCK caused the greatest decrease in c-Met protein levels and is a physiological stimulant of pancreatic acinar cell function by binding and activating its corresponding GPCR receptor, the CCKα receptor (41, 64). The ability of CCK to alter c-Met protein levels showed similarities as well as differences in the ability of HGF to alter c-Met levels in pancreatic acini as well as other cells. Activation of the CCKα receptor by CCK-8 in rat pancreatic acini caused rapid c-Met receptor down-regulation. The effect of CCK was rapid in that it was detectable within 10 min and reached a maximum effect after 45 min. This stands in contrast to our own findings of HGF-induced c-Met down-regulation in pancreatic acini as well as in other cells and of c-Met down-regulation caused by other stimuli in other cells, where a much longer stimulation time was needed to cause detectable changes in c-Met protein levels (6, 12, 13, 53–56). Specifically, in contrast to CCK-induced rapid c-Met down-regulation, in pancreatic acinar cells HGF incubations of more than 3 h were needed to detect a decrease in c-Met receptor levels. Similarly, in HeLa and HEK293T cells changes in c-Met protein level were only observed after 1 h of HGF incubation, and a maximum effect was seen after 4 h (54, 55), although in LMS-1, RD, A549, and A431 cells HGF incubation times of more than 4 h were needed to cause c-Met down-regulation (56). In T47D cells transfected with c-Met, the first effects of HGF stimulation on c-Met were seen after 30 min, while 2 h of incubation were needed to reduce c-Met receptor levels by 75% (53). Furthermore, in a study of interferon-α-induced c-Met down-regulation in primary human hepatocytes, 24 h of incubation were needed to reduce c-Met levels (12), and 6 h of H2O2 stimulation were needed to cause detectable c-Met down-regulation in MIMCD-3 cells (13). Therefore, our results demonstrate that CCK-induced c-Met down-regulation occurred much faster than HGF-induced c-Met down-regulation in acini or c-Met down-regulation induced by HGF or other stimuli in other cells.

In pancreatic acini the CCKα receptor subtype has been shown to exist in both a low affinity and a high affinity receptor state, each of which can activate distinct downstream signaling cascades (16, 39, 65–67). A number of our findings support the conclusion that CCK-induced c-Met down-regulation is mediated by the low affinity state but not the high affinity state of the CCKα receptor.

Effects of CCK on c-Met levels were studied in greater detail. CCK is a gastrointestinal hormone as well as a neurotransmitter that influences pancreatic acinar cell function by binding and activating its corresponding GPCR receptor, the CCKα receptor (41, 64). The ability of CCK to alter c-Met protein levels showed similarities as well as differences in the ability of HGF to alter c-Met levels in pancreatic acini as well as other cells. Activation of the CCKα receptor by CCK-8 in rat pancreatic acini caused rapid c-Met receptor down-regulation. The effect of CCK was rapid in that it was detectable within 10 min and reached a maximum effect after 45 min. This stands in contrast to our own findings of HGF-induced c-Met down-regulation in pancreatic acini as well as in other cells and of c-Met down-regulation caused by other stimuli in other cells, where a much longer stimulation time was needed to cause detectable changes in c-Met protein levels (6, 12, 13, 53–56). Specifically, in contrast to CCK-induced rapid c-Met down-regulation, in pancreatic acinar cells HGF incubations of more than 3 h were needed to detect a decrease in c-Met receptor levels. Similarly, in HeLa and HEK293T cells changes in c-Met protein level were only observed after 1 h of HGF incubation, and a maximum effect was seen after 4 h (54, 55), although in LMS-1, RD, A549, and A431 cells HGF incubation times of more than 4 h were needed to cause c-Met down-regulation (56). In T47D cells transfected with c-Met, the first effects of HGF stimulation on c-Met were seen after 30 min, while 2 h of incubation were needed to reduce c-Met receptor levels by 75% (53). Furthermore, in a study of interferon-α-induced c-Met down-regulation in primary human hepatocytes, 24 h of incubation were needed to reduce c-Met levels (12), and 6 h of H2O2 stimulation were needed to cause detectable c-Met down-regulation in MIMCD-3 cells (13). Therefore, our results demonstrate that CCK-induced c-Met down-regulation occurred much faster than HGF-induced c-Met down-regulation in acini or c-Met down-regulation induced by HGF or other stimuli in other cells.

In pancreatic acini the CCKα receptor subtype has been shown to exist in both a low affinity and a high affinity receptor state, each of which can activate distinct downstream signaling cascades (16, 39, 65–67). A number of our findings support the conclusion that CCK-induced c-Met down-regulation is mediated by the low affinity state but not the high affinity state of the CCKα receptor. The effects of CCKs on c-Met levels first were detectable at 1 nM, and a maximal effect was seen at 30 nM, a CCK concentration that coincides with CCK occupation of the low affinity state of the CCKα receptor reported by binding studies (68, 69). Furthermore, CCK-JMV, which is an agonist of the high affinity state and an antagonist of the low affinity state of the CCKα receptor in rat pancreatic acini (70), did not cause c-Met receptor down-regulation. These results demonstrate that the ability of CCKα receptor activation to cause c-Met receptor down-regulation is similar to its ability to stimulate tyrosine phosphorylation of the PKC isoform PKC8 (71), to cause down-regulation of the GPCR for bombesin-related peptides, or to stimulate desensitization of m1 muscarinic cholinergic receptors in pancreatic acini (72), which are all mediated solely by activation of the low affinity state of the CCKα receptor (73). These results differ from the ability of CCK to stimulate tyrosine phosphorylation of p125FAK, paxillin, and PyK2 (74), which depends on activation of both the high and low affinity state of the CCKα receptor or from the ability of CCK to

FIGURE 16. Effect of CCK-8 on c-Met receptor levels in rat pancreatic acini in vivo. Adult male Wistar rats were injected intraperitoneally with 10 μg/kg CCK-8 dissolved in sterile saline solution at 20-min intervals for the times indicated. After CCK-8 treatment, rats were euthanized, and pancreatic acini were prepared as described under “Experimental Procedures.” Aliquots of dispersed acini were lysed; equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Upper panel, representative blot from one of four independent experiments is shown. Top upper panel, membranes were analyzed using mouse monoclonal anti-c-Met antibody. Top lower panel, to ensure equal loading of protein, membranes were re-probed with goat polyclonal anti-β-actin antibody. Bands were visualized using chemiluminescence. Representative blots from four independent experiments for each time point are shown. Bottom, the graph shows data from band intensity measurements (mean ± S.E.) from four independent experiments. Values are expressed as percentage of control c-Met levels (i.e. no CCK-8 treatment).

CCK-stimulated c-Met Down-regulation

Time Post CCK-8 (min)

| Time Post CCK-8 (min) | WB:αc-Met | WB:αβ-Actin |
|----------------------|-----------|-------------|
| 0                    |           |             |
| 20                   |           |             |
| 40                   |           |             |

CCK-8 (20 min) CCK-8 (40 min)

p = 0.0001

p = 0.0011
stimulate down-regulation of the insulin receptor, which is mediated by activation of only the high affinity CCK<sub>A</sub> receptor state (75). These results demonstrate that not only are activations of phospholipase A<sub>2</sub> and D pathways but not activation of PLC (66, 67, 76). In contrast, activation of the low affinity CCK<sub>A</sub> receptor leads to PLC activation, which in turn results in the formation of diacylglycerol and inositol 1,4,5-trisphosphate (66, 67, 76). Diacylglycerol binds and activates PKCs, whereas inositol 1,4,5-trisphosphate causes an increase in intracellular calcium levels, which in turn causes the activation of calcium-dependent kinases in pancreatic acini (16, 39, 77). A number of our findings support the conclusion that following CCK-independent kinases in pancreatic acini (16, 39, 77). A number of our findings support the conclusion that following CCK-induced stimulation of the low affinity state of the CCK<sub>A</sub> receptor, c-Met down-regulation is at least partially mediated by activation of both limbs of the PLC cascade (i.e. PKC activation and cytosolic calcium changes). First, inhibiting PKC activation by GF109203X resulted in a 20% inhibition of the ability of CCK to stimulate c-Met down-regulation. Previous studies demonstrated that under the experimental conditions used, PKC-mediated tyrosine phosphorylation of p125<sup>FAK</sup> and paxillin induced by TPA was completely inhibited (62), and under the same conditions with a calcium-free medium, complete inhibition of CCK activation of PyK2 tyrosine phosphorylation occurred (46). Therefore, these results suggest PKC-dependent and PKC-independent mechanisms are involved in c-Met down-regulation caused by CCK. Second, inhibition of CCK-stimulated increases in intracellular calcium by thapsigargin inhibited CCK-induced c-Met down-regulation by 33%. Because the experimental conditions used cause complete inhibition of CCK-stimulated increases in cellular calcium (74, 78), this partial inhibition by thapsigargin supports the conclusion that calcium-independent pathways activated by CCK also contribute to c-Met down-regulation. Third, because CCK stimulation of a number of cellular changes demonstrate a potentiated effect of the activation of both limbs of the PKC cascade (74), the inhibition of both PKC activation and increases in cytosolic calcium induced by CCK was examined. This resulted in a 61% inhibition of CCK-induced c-Met down-regulation supporting the conclusion that 39% of the effect of CCK on c-Met were mediated by a PLC-independent mechanism. Fourth, the importance of PKC activation in mediating c-Met down-regulation was supported by our finding that both carbachol and bombesin, which activate PLC in pancreatic acini (16, 79, 80), but not vasoactive intestinal peptide and secretin, whose actions are mediated by adenylate cyclase (16, 81), also caused significant c-Met down-regulation. These findings support the conclusion that the ability of CCK to induce c-Met down-regulation is mediated by both PLC-independent and PLC-dependent mechanisms and that the latter involves activation of PKC- and calcium-dependent mechanisms.

Similar to a number of other GPCR and growth factor receptors, c-Met receptor down-regulation is reported in other cells to result in a decreased cellular response by the receptor agonists (11, 47, 53). In pancreatic acini, CCK preincubation completely blocked the ability of HGF to stimulate tyrosine phosphorylation of tyrosines 1234 and 1235 of the c-Met receptor, which have been shown to be essential for the activation of c-Met’s tyrosine kinase (82). Furthermore, CCK preincubation inhibited HGF-stimulated tyrosine phosphorylation of the adaptor protein Gab1 (83), a major substrate of the activated c-Met receptor, which has been shown previously to be tyrosine-phosphorylated after HGF stimulation in rat pancreatic acini (18, 43). Because of the similar time courses to loss of the c-Met, this decreased c-Met and GAB tyrosine phosphorylation is probably because of the disappearance of the c-Met from the cell surface with CCK treatment. Studies in other cells have shown that HGF stimulation can lead to the PKC- and calcium-dependent phosphorylation of serine 985 of the c-Met receptor that results in the inactivation of c-Met’s tyrosine kinase (7). We found that CCK stimulation alone caused phosphorylation of serine 985 of c-Met and that CCK potentiated HGF-induced phosphorylation of serine 985 of c-Met. In light of the effect of serine 985 phosphorylation on c-Met tyrosine kinase activity reported in other cells (7), our results suggest this inactivation mechanism for the c-Met tyrosine kinase also could possibly be participating in the inhibitory effects on c-Met kinase activity in pancreatic acini triggered by CCK in addition to the c-Met down-regulation. The results discussed above demonstrate that CCK not only causes a marked decrease in c-Met protein levels but also blocks the biological cellular responses of HGF, thereby preventing the activation of signaling cascades downstream of the c-Met receptor. Aberrant c-Met signaling has been shown to be important in numerous neoplasias of the GI tract, including pancreatic cancer (8–10, 20, 84), and receptors for CCK/gastrin, muscarinic cholinergic agents, and bombesin-related peptides are frequently expressed by these tumors. As our data provide strong evidence that CCK-8 can down-regulate c-Met expression in Panc-1 pancreatic cancer cells, the specific role of the c-Met receptor in the future.

In different cells several mechanisms of c-Met receptor down-regulation have been described (6, 12, 13, 15, 53–56, 85, 86). In various cells HGF-induced c-Met down-regulation is dependent on c-Met receptor ubiquitination mediated by the ubiquitin-protein ligase c-Cbl, and the HGF-induced down-regulation can be blocked by inhibiting the proteasome and by inhibiting late lysosomes (53–56). There are also reports that by activating other receptor systems like the Notch receptor in breast cancer cells, the interferon-α receptor, and the transforming growth factor-β1 receptor in primary human hepatocytes, and by oxidative stress in renal cells, it is possible to alter c-Met expression at the transcriptional level (6, 12, 13). Finally, studies demonstrate in various cells that stimulating with sumarin, EGF, or lysophosphatidic acid results in shedding of the extracellular domain of the c-Met receptor, a process that is mediated by extracellular metalloproteinases and that leads to rapid degradation of the remaining transmembrane domain (15, 85, 86). Our results show that CCK-induced c-Met down-regulation differs from mechanisms described in the literature.
CCK-stimulated c-Met Down-regulation

in a number of ways and that this likely demonstrates a new and unique mechanism of c-Met receptor down-regulation. First, as discussed above, CCK-8-induced c-Met down-regulation was much more rapid than reports of c-Met down-regulation induced by HGF or other stimuli in the literature to date or of HGF-induced down-regulation in pancreatic acini (6, 12, 13, 53–56). Second, in contrast to down-regulation of c-Met by HGF, which depends on ubiquitination of c-Met (55, 56), we did not find ubiquitination of c-Met after CCK stimulation. Third, inhibition of the proteasome by lactacystin, which in other tissues and in pancreatic acini prevents HGF-induced c-Met down-regulation (11, 54–56), did not block the decrease of c-Met levels observed after CCK incubation, showing that the proteasome was not involved in the action of CCK on c-Met. Fourth, whereas in pancreatic acini and in HeLa cells inhibition of the lysosome by concanamycin A only minimally inhibited HGF-induced c-Met down-regulation (55), lysosome inhibition had a marked effect in pancreatic acini after CCK stimulation, causing c-Met levels to return to 71% of control levels. Fifth, it is unlikely that CCK-induced c-Met down-regulation is regulated on a transcriptional level, because in studies that reported changes in c-Met protein levels caused by transcriptional mechanisms much longer stimulation times were needed (12–14, 57). Finally, the fact that in our study inhibitors of endocytosis as well as inhibition of the lysosome could block the down-regulation of the extracellular domain of the c-Met receptor after CCK stimulation and our finding that incubation with CCK-8 for a short period of time induces an increase in c-Met internalization speak against CCK-induced shedding of the extracellular c-Met domain as a possible explanation for the effect seen in pancreatic acini.

In conclusion our study for the first time shows that GPCRs activated by some GI hormones/neurotransmitters are able to rapidly down-regulate c-Met levels. CCK stimulation not only caused rapid, time-, and dose-dependent down-regulation of the c-Met receptor, but this resulting down-regulation also blocked the ability of HGF to alter cellular activity by preventing HGF-induced phosphorylation of the receptor and of downstream signaling pathways. The effect of CCK was observed not only in vitro in dispersed rat pancreatic acini, in Panc-1 cells, and in single acinar cells but also in vivo after intraperitoneal CCK injection in the rat model. The effect of CCK was mediated by activation of the low affinity receptor state of the CCK₇ receptor and involved both activation of PLC-dependent and PLC-independent mechanisms with activation of both PKC and intracellular calcium-induced pathways. Although our findings of HGF-induced c-Met degradation in rat pancreatic acini agree with reports in the literature, CCK-induced c-Met down-regulation shows important differences in the mechanism involved, suggesting a new and unique pathway of c-Met degradation, which is because of rapid clathrin-dependent internalization of the c-Met receptor and subsequent degradation of the receptor by cellular processes that are independent from receptor ubiquitination and proteasome function, but require the lysosome pathway. Understanding this new mechanism of influencing c-Met expression could be important for understanding its regulation in normal tissue as well as for influencing aberrant c-Met expression and signaling in pancreatic cancer and other malignancies.

REFERENCES

1. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. (2003) Nat. Rev. Mol. Cell Biol. 4, 915–925
2. Rosario, M., and Birchmeier, W. (2003) Trends Cell Biol. 13, 328–335
3. Krause, D. S., and Van Etten, R. A. (2005) N. Engl. J. Med. 353, 172–187
4. Cooper, C. S., Park, M., Blair, D. G., Tainsky, M. A., Huebner, K., Croce, C. M., and Vande Woude, G. F. (1984) Nature 311, 29–33
5. birchmeier, C., and Gherardi, E. (1998) Trends Cell Biol. 8, 404–410
6. Stella, M. C., Trusolino, L., Pennacchietti, S., and Comoglio, P. M. (2005) Mol. Cell. Biol. 25, 3982–3996.
7. Hashigasako, A., Machide, M., Nakamura, T., Matsumoto, K., and Naka-mura, T. (2004) J. Biol. Chem. 279, 26445–26452
8. Christensen, J. G., Burrows, J., and Salgia, R. (2005) Cancer Lett. 225, 1–26
9. Croso, S., Comoglio, P. M., and Giordano, S. (2005) Trends Mol. Med. 11, 284–292
10. Furukawa, T., Duguig, W. P., Kobari, M., Matsuno, S., and Tsao, M. S. (1995) Am. J. Pathol. 147, 889–895
11. Hammond, D. E., Carter, S., and Clague, M. J. (2004) Curr. Top. Microbiol. Immunol. 286, 21–44
12. Radeva, S., Jaruga, B., Hong, F., Kim, W. H., Fan, S., Cai, H., Strom, S., Liu, Y., El-Assal, O., and Gao, B. (2005) Gastroenterology 122, 1020–1034
13. Zhang, X., Yang, J., Li, Y., and Liu, Y. (2005) Am. J. Physiol. 288, F16–F26
14. Zhang, X., and Liu, Y. (2003) Am. J. Physiol. 284, F1216–F1225
15. Nath, D., Williamson, N. J., Jarvis, R., and Murphy, G. (2001) J. Cell Sci. 114, 1213–1220
16. Jensen, R. T. (1994) in Physiology of the Gastrointestinal Tract (Johnson, L. R., Jacobson, E. D., Christensen, J., Alpers, D. H., and Walsh, J. H., eds) 3rd Ed., pp. 1377–1446, Raven Press, Ltd., New York
17. Herynk, M. H., Stoeltzing, O., Reinhnm, N. Parikh, N. U., Abounader, R., Latcero, J., Radinsky, R., Ellis, L. M., and Gallicco, G. E. (2003) Cancer Res. 63, 2990–2996
18. Hoffmann, K. M., Tapia, J. A., and Jensen, R. T. (2005) Gastroenterology 128, A631
19. Resnick, M. B., Routhier, I., Konkin, T., Sabo, E., and Pricolo, V. E. (2004) Clin. Cancer Res. 10, 3069–3075
20. Kiehn, K., Herzog, K. H., and Folsch, U. R. (1997) Pancreas 15, 35–40
21. Kim, J., Ahn, S., Guo, R., and Daaka, Y. (2003) Biochemistry 42, 2887–2894
22. Keely, S. J., Uribe, J. M., and Barrett, K. E. (1998) J. Biol. Chem. 273, 27111–27117
23. Prenzel, N., Zwick, E., Daub, H., Lesser, M., Abraham, R., Wallcasch, C., and Ullrich, A. (1999) Nature 402, 884–888
24. Aparicio, I. M., Garcia-Marin, L. J., Andreolotti, A. G., Bodega, G., Jensen, R. T., and Bradamson, M. J. (2005) Biochim. Biophys. Acta 1643, 37–46
25. Vila, M. R., Nakamura, T., and Real, F. X. (1995) Lab. Invest. 73, 409–418
26. Paciucci, R., Vila, M. R., Adell, T., Diaz, V. M., Tora, M., Nakamura, T., and Real, F. X. (1998) Am. J. Pathol. 153, 201–212
27. Lefebvre, V. H., Otonkoski, T., Ustino, J., Huotari, M. A., Pipeleers, D. G., and Bouwens, L. (1998) Diabetes 47, 134–137
28. Gahr, S., Merger, M., Bollheimer, L. C., Hammerschmidt, C. G., Scholmerich, J., and Hugl, S. R. (2002) J. Mol. Endocrinol. 28, 99–110
29. Di Renzo, M. F., Olivero, M., Giamoconi, A., Porle, H., Chastre, E., Miros-say, L., Nordlinger, B., Brett, S., Boddart, S., Giordano, S., Plebani, M., Gespach, C., and Comoglio, P. M. (1995) Clin. Cancer Res. 1, 147–154
30. Ebert, M., Yokoyma, M., Friess, H., B€uchler, M. W., and Korc, M. (1994) Cancer Res. 54, 5775–5778
31. Furukawa, M., Raffeld, M., Mateo, C., Sakamoto, A., Moody, T. W., Ito, T., Venzon, D. J., Serrano, J., and Jensen, R. T. (2005) Cancer Res. 65, 3233–3242
32. Calvo, E. L., Boucher, C., Pelletier, G., and Morissette, J. (1996) Biochem. Biophys. Res. Commun. 229, 257–263
33. Otte, J. M., Schwenger, M., Bruneke, G., Sparmann, G., Emmrich, J., Schnitz, F., Folsch, U. R., and Herzog, K. H. (2001) Eur. J. Clin. Investig. 31, 865–875
34. Menke, A., Yamaguchi, H., Giehl, K., and Adler, G. (1999) Pancreas 18,
