PKC, p42/p44 MAPK, and p38 MAPK are required for HGF-induced proliferation of H441 cells

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In this paper, we studied the signaling pathway used by hepatocyte growth factor/scatter factor (HGF) to stimulate mitosis. We show, using H441 cells, that 1) HGF activates membrane-associated protein kinase C (PKC); the activity is transient and peaks within 30 min; 2) HGF activates p42/p44 and p38 mitogen-activated protein kinases (MAPKs); maximum activity in both is within 10 min; and 3) the activation of neither p38 nor p42/p44 MAPK is dependent on PKC, indicating that HGF uses separate and nonintersecting pathways to activate these two classes of kinase. However, phorbol 12-myristate 13-acetate also activates both MAPKs as well as PKC, but this activation is abolished in cells pretreated with the PKC inhibitor GF-109203X. HGF was found to significantly increase [3H]thymidine incorporation within 5 h; peak thymidine incorporation was observed at 16 h. However, when cells were pretreated with inhibitors of p42/p44 (PD-98059), p38 (SB-203580), or PKC (GF-109203X, Gö-6983, or myristoylated inhibitor peptide19,27), HGF-induced thymidine uptake was diminished in a dose-dependent manner. Taken together, these results demonstrate that HGF activates PKC and both MAPKs simultaneously through parallel pathways and that the activation of the MAPKs does not depend on PKC. However, p38 and p42/p44 MAPKs and PKC may all be essential for HGF-induced proliferation of H441 cells.

hepatocyte growth factor; mitogen-activated protein kinase; lung injury; protein kinase C; extracellular signal-regulated kinase
p42/p44 MAPK and report that HGF also activates p38 MAPK with an efficiency and a time course similar to those found for the activation of p42/p44. We also show that HGF activates PKC concurrent with its activation of the MAPKs but that neither the activation of p42/p44 nor p38 MAPK is dependent on PKC activity. The inhibition of either MAPK or PKC results in an inhibition of the DNA synthesis stimulated by HGF, suggesting that p38 and p42/p44 MAPKs and PKC are all required for HGF-stimulated mitosis in these cells.

**METHODS.**

*Materials.* We obtained radioactive materials from NEN (Boston, MA). Bisindolylmaleimide I (GF-109203X), G-6983, myristoylated PKC inhibitor peptide<sub>19-27</sub> (M-PKC), PD-80509, SB-203580, phorbol 12-myristate 13-acetate (PMA), protein A-agarose, PKA inhibitor peptide<sub>25-24</sub> and PKC-δ-specific substrate were from Calbiochem-Novabiochem (San Diego, CA). Biotinylated PKC-ζ-specific substrate was from Genzyme (Cambridge, MA). Myelin basic protein 4–14 (MBP<sub>4-14</sub>) fragment was from Sigma (St. Louis, MO). Antibodies to the c-Met protein, PKC isoforms, phosphotyrosine, and anti-rabbit IgG-horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to the p38 and p42/p44 MAPKs and their phosphorylated forms were from New England BioLabs (Beverly, MA). HGF was from R&D Systems (Minneapolis, MN). The protein assay kit was from Pierce (Rockford, IL). Other reagents were from Sigma.

*Cell culture.* We obtained the human lung adenocarcinoma cell line H441 from the American Type Culture Collection (Manassas, VA). We maintained the cells in McCoy’s 5A medium (JRH Biosciences, Lenexa, KS) supplemented with 10% (vol/vol) fetal bovine serum (GIBCO BRL, Life Technologies, Gaithersburg, MD) and 50 μg/ml of gentamicin antibiotic in standard tissue culture flasks. Before treatment with growth factor, we deprived the cells of serum for 16–24 h. Confluence of the cells was maintained at 60–80%.

*Immunoprecipitation of c-Met receptor and Western blotting.* We treated H441 cells with HGF at a dose of 10 ng/ml for various times. After the stipulated time periods, we scraped the cells into an ice-cold buffer containing Tris-HCl (25 mM, pH 7.5), EDTA (2 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), leupeptin (10 μg/ml), pepstatin (10 μg/ml), sodium orthovanadate (0.2 mM), NaF (50 mM), and NaCl (150 mM). After keeping the lysates on ice for 30 min, we centrifuged them at 14,000 rpm for 15 min and collected the supernatants in fresh tubes. For immunoprecipitation, we incubated ~1 mg of protein with anti-c-Met antibody (1–2 μg) at 4°C with constant rotation for 2 h. We captured the immune complexes in 20 μl of a 1:1 slurry of protein A-agarose. To eliminate nonspecifically bound proteins, we washed the immunoprecipitates twice with lysis buffer and once each with 1 M NaCl, 0.05 M Tris (pH 6.8), and Tris-saline-azide (pH 8.0). To elute the bound protein, we boiled the immunoprecipitates for 5 min in 50–75 μl of SDS-PAGE sample buffer containing β-mercaptoethanol. Equal amounts of sample protein were loaded on 10% polyacrylamide gels, and the proteins were resolved by SDS-PAGE. After separation, we transferred the protein to polyvinylidene difluoride (PVDF) membrane and performed immunoblotting with the appropriate antibody for 2–3 h. For chemiluminescence detection with the enhanced chemiluminescence kit (ECL, Amersham, Arlington Heights, IL), we used HRP-conjugated anti-rabbit IgG antibody.

*In vitro assay for protein kinase C.* We washed the cells twice with ice-cold PBS and seeded them into 200 μl of homogenization buffer [20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.5 mM EDTA, 0.5 mM EGTA, 25 μg/ml of leupeptin, 25 μg/ml of pepstatin, 1 mM PMSF, and 0.2 mM sodium orthovanadate]. We homogenized the cells and centrifuged the homogenate at 40,000 rpm for 30 min at 4°C (Optima TLX Ultracentrifuge, Beckman, Palo Alto, CA). The membrane pellet was dissolved in 100 μl of homogenization buffer containing 0.05% Triton X-100, kept on ice for 30 min, and centrifuged again as above. For kinase activity, we incubated the cytosolic and solubilized membrane fractions (10 μl each) with 2 μg of MBP<sub>4-14</sub>, 0.8 μg/ml of olein, 8 μg/ml of phosphatidylinerseine, 20 μM ATP, and 1 μCi of [γ-<sup>32</sup>P]ATP in 50 μl of kinase buffer [20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 1 mM Ca<sub>Cl</sub><sub>2</sub>, and 50 μg/ml of leupeptin]. The reaction was allowed to proceed for 15 min at 30°C. We applied 10 μl of the reaction mixtures to phosphocellulose paper disks (Life Technologies, Grand Island, NY) and washed the disks three times with 1% acetic acid and twice with water before counting the radioactivity in a scintillation counter. The counts were normalized with the amount of protein in the reaction mixture. MBP<sub>4-14</sub> has been shown to be a specific substrate for PKC that reacts broadly with most isoforms of PKC, although its reactivity with PKC-δ and ζ is relatively weak (8, 40). The specificity of the assay was confirmed by assaying PKC activation in the presence and absence of lipid activators and CAMP-dependent protein kinase inhibitor peptide<sub>5-24</sub>.

To attribute the increased kinase activity to a particular group of PKC isoforms, we also carried out the kinase assay after immunoprecipitating the α, βII, δ, and ζ isoforms. Overnight, we immunoprecipitated ~200–300 μg of membrane protein with antibodies specific to PKC isoforms, together with protein A-agarose, in buffer A containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1.5 mM Ca<sub>Cl</sub><sub>2</sub>, 10 mM NaF, 1 mM PMSF, 10 μg/ml of leupeptin, and 10 μg/ml of pepstatin. We washed the immune complexes once with buffer A and twice with 50 mM Tris-HCl, pH 7.5. We resuspended the complexes in 50 mM Tris-HCl, pH 7.5, with 5 mM β-mercaptoethanol. We initiated the kinase reaction by adding kinase buffer. The final reaction mixture contained 0.8 μg/ml of olein, 8 μg/ml of phosphatidylinerseine, 20 μM ATP, 1 μCi of [γ-<sup>32</sup>P]ATP, and 3 μg of peptide substrate (MBP<sub>4-14</sub>, δ-specific substrate, and ζ-specific biotinylated substrate for conventional, δ, and ζ isoforms, respectively). The reaction was terminated by applying the supernatant to phosphocellulose paper. The rest of the method was the same as that used for total kinase activity.

*p42/p44 MAPK and p38 MAPK activation.* To study HGF-induced MAPK activation, we seeded H441 cells in 60-mm tissue culture dishes and treated them with HGF (10 ng/ml) or PMA (200 nM) for various time periods. At the end of the incubation, we washed the cell layer twice with PBS and lysed the cells in 200 μl of Tris-HCl buffer, pH 7.4, containing 25 mM Tris, 1% Igepal CA-630 (Sigma), 150 mM NaCl, 50 mM NaF, 200 μM sodium orthovanadate, and 1 mM PMSF. We probe-sonicated the lysates for 5 s and centrifuged them at 14,000 rpm for 10 min to remove the cell debris. After separating the protein on 10% SDS-polyacrylamide gels, we transferred it to a PVDF membrane and blotted the membrane with anti-phospho-specific p42/p44 MAPK and anti-phospho-specific p38 MAPK antibodies. Blotting the membranes with rat anti-p42 MAPK and anti-p38 MAPK antibodies confirmed equal protein loading in the gels. Enhanced chemiluminescence detected the proteins of interest.
We used the MAPK antibodies at a 1:1,000 dilution in Tris-buffered saline, containing 5% BSA and 0.2% Tween 20. Anti-rabbit IgG-HRP secondary antibody was used at a 1:5,000 dilution in the same buffer.

To explore the role of PKC in the activation of MAPKs by HGF, we treated serum-starved H441 cells with a PKC inhibitor, GF-109203X (10 μM for 1 h), or depleted PKC by treating the cells with 200 nM PMA for 16–20 h. GF-109203X inhibits PKC by competing with ATP for its binding site on PKC (31), whereas prolonged treatment with PMA translocates PKC from the plasma membrane to the cytosol and thus inactivates it (34).

Thymidine incorporation assays. We studied the induction of cell proliferation by measuring the incorporation of [methyl-3H]thymidine into newly synthesized DNA. We seeded the cells in 24-well plates to grow for 24 h and then deprived the cells of serum overnight. We pretreated the cells for 1 h with either PD-98059, SB-203580, GF-109203X, Go6983, or M-PKCI (all 0.1–10 μM). We stimulated the cells with HGF for 16 h and added 0.5 μCi of [methyl-3H]thymidine to all wells during the last hour of incubation. After the stipulated times, we washed the cells twice with ice-cold PBS, precipitated the DNA by washing the cell layer twice with 5% trichloroacetic acid, and dissolved it in 0.1 N NaOH for scintillation counting. We chose the 16-h time point for data analysis after ascertaining the time course of HGF-induced thymidine uptake.

Miscellaneous methods. For protein estimation in the samples, we used the bicinchoninic acid method according to the manufacturer’s instructions (Pierce, Rockford, IL). We performed densitometric analysis with a molecular imaging system (Bio-Rad, Hercules, CA). Statistical analysis for significance was performed by ANOVA with StatView II software (Abacus Concepts, Berkeley, CA).

RESULTS

Expression and phosphorylation of c-Met receptor in H441 cells. To confirm that the c-Met receptor is present and responds to HGF in H441 cells, we immunoprecipitated c-Met from cell lysates with anti-c-Met antibody and examined it by Western blot analysis (Fig. 1A). The 145-kDa band corresponds to the β-chain of mature c-Met, and the 170-kDa band is an intracellular single-chain precursor of c-Met. Western blotting with anti-phosphotyrosine antibody showed that the β-chain of the c-Met receptor was phosphorylated in response to HGF. A significant amount of constitutive c-Met phosphorylation was also present in untreated cells. As is evident from Fig. 1B, the phosphorylation was increased within minutes of HGF addition and was dependent on the duration of treatment. Maximum activation occurred within 5 min, and thereafter, phosphorylation declined. This decrease in phosphorylation may have resulted from the downregulation and degradation of the c-Met receptor (21) or the phosphotyrosine phosphatase activity associated with the activated receptor (32).

HGF stimulates PKC activity. The c-Met receptor has been shown to be downregulated by the activation of PKC by PMA (9); however, the induction of PKC activity by HGF has not been reported. In this study, we show that HGF transiently activates membrane-associated PKC activity. All three PKC groups, i.e., conventional, novel, and atypical PKCs, are represented in H441 cells as determined by Western blotting with antibodies specific to PKC-α, -βII, -δ, and -ζ isoforms (Fig. 2A). We carried out in vitro kinase activity assays with a MBP4–14 fragment as a substrate for PKC. Figure 2B shows the time course of membrane-associated kinase activity in response to HGF and PMA. HGF induced an increase in membrane-bound kinase activity (~170%), which peaked at 30 min. In comparison, PMA induced a rapid and somewhat larger increase (~250%) in membrane activity that was sustained through at least 1 h. To eliminate the possibility of a change in PKC activity in cells in culture that is independent of growth factor treatment, we included control cells at various time points. There was no increase in membrane-associated kinase activity in normal control cells (data not shown).

We also performed kinase assays by specifically immunoprecipitating PKC-α, -βII, -δ, and -ζ isoforms from the membrane protein after the cells were treated with HGF for 10 min. All the isoforms were activated by HGF: α, 1.7-fold; βII, 2-fold; δ, 1.6-fold; and ζ,
1.3-fold compared with time-matched controls (Fig. 2C). The activation of all the isoforms except PKC-ζ was significant ($P < 0.05$).

**HGF activates p42/p44 MAPK (extracellular signal-regulated kinase 2/1) and p38 MAPK.** We used antibodies to site-specific phosphorylated forms of p42/p44 and p38 MAPKs to estimate changes in their activity induced by HGF treatment. The results are shown in Fig. 3. There was significant phosphorylation of both MAPKs in resting and starved cells; this was increased over the basal level by HGF. The change in phosphorylation of p42/p44 was observed as early as 1 min, peaked to ~2-fold of basal level within 5–10 min, and stayed above basal level for at least 1 h of observation, although the activation after 10 min was not significant (Fig. 3B, top). HGF also stimulated the phosphorylation of p38 MAPK (Fig. 3A, bottom). This activation was also rapid and was ~3-fold over basal levels, and the time course was similar to that of p42/p44. Like p42/p44, p38 peaked at 5–10 min, but the phosphorylation was completely reversed somewhat more quickly, i.e., within 30 min (Fig. 3B, bottom). The corresponding Western analyses for total p42 and p38 protein showed approximately equal amounts in all samples (Fig. 3A).

**The pathways used by HGF to activate p42/p44 and p38 are independent of PKC.** We first determined that activation of PKC by PMA results in the activation of both p42/p44 and p38 MAPKs. H441 cells were given 200 nM PMA, and MAPK phosphorylation was measured after 10 min. PMA induced a two- to threefold increase in phosphorylation of both MAPKs (Fig. 4A), comparable to that induced by 10 ng/ml of HGF. Because pretreatment with GF-109203X abolished activation of both MAPKs by PMA, the pathway used by PMA is dependent on PKC. However, the pathways used by HGF are independent of PKC because pretreatment with GF-109203X for 1 h did not affect the response to HGF. We also found that the basal level of phosphorylation of p42/p44 MAPK and p38 MAPK was reduced by GF-109203X (Fig. 4B), but the reduction was not significant.

To confirm these findings, we treated the cells with PMA for 16–20 h before adding HGF or PMA (Fig. 4C). Overnight PMA treatment reduced the basal level of p42/p44 phosphorylation; surprisingly, it elevated p38 basal activity, but the change was not significant (Fig. 4C). Because of this increase in basal p38 activation, we could not clearly discern the effects of HGF and a 10-min PMA treatment. However, the effect on p42/p44 was similar to that obtained with GF-109203X; PMA pretreatment abrogated the acute stimulatory effects

Fig. 2. Protein kinase C (PKC) in H441 cells. A: isoforms of PKC detected by Western analysis of H441 cell lysates. Cellular protein (~5–10 μg) was electrophoresed on a 10% gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and immunoblotted with a 1:1,000 dilution of antibodies specific to the individual PKC isoforms. B: time-dependent activation of PKC in the membrane and cytosolic fractions of H441 cells in response to 10 ng/ml of HGF (top) and 200 nM phorbol 12-myristate 13-acetate (PMA; bottom). PKC activity in the fractions was assessed by an in vitro kinase assay with myelin basic protein (MBP) as a substrate in the presence of $[^{32}P]ATP$. Results are averages ± SE of at least 3 separate experiments. *$P < 0.05$ compared with time-matched controls or time 0 (100%). C: kinase activity of specific PKC measured at 10 min. Activity was determined after membrane-bound PKC was immunoprecipitated with isoform-specific antibodies. Kinase assay was done as described in METHODS. Results are averages of 2 separate experiments done in duplicate. *$P < 0.05$ compared with control.
of PMA but did not affect the activation of either MAPK by HGF. Taken together, the results indicate that PMA activates p42/p44 MAPK and p38 MAPK via a PKC-dependent pathway; HGF acts through an alternate mechanism independent of PKC.

HGF-induced increase in DNA synthesis depends on the activation of both p42/p44 and p38 MAPKs and PKC. The time course of the HGF-induced increase in thymidine incorporation is shown in Fig. 5A. Addition
of HGF (10 ng/ml) to the culture medium stimulated [3H]thymidine uptake in H441 cells by ~1.6-fold at 16 h. To investigate the role of p42/p44 and p38 MAPKs in H441 cell proliferation, we used specific inhibitors of p42/p44 and p38, PD-98059 and SB-203580, respectively. PD-98059 is an inhibitor of MAPK kinase (MEK) and, therefore, inhibits activation of p42/p44 (7). SB-203580 inhibits the phosphory-

Fig. 5. Inhibitors of MAPK kinase (MEK), p38 MAPK, and PKC suppress HGF-induced thymidine uptake. A: time course of HGF-induced thymidine uptake. Cells were stimulated with HGF (10 ng/ml) for various times, and 0.5 μCi of [3H]thymidine were added during the last hour of incubation. *P < 0.05 vs. time-matched control. $P < 0.1 vs. control. B–F: dose-dependent inhibition of HGF-induced thymidine uptake by MEK inhibitor PD-98059, p38 inhibitor SB-203580, and PKC inhibitors GF-109203X (BIM I), Go-6983, and myristoylated inhibitor peptide 19–27 (M-PKCI), respectively. Data are means ± SE of at least 3 separate experiments. Inhibitors were added 1 h before addition of HGF for 16 h. *P < 0.05 vs. HGF. ¥P < 0.05 vs. control. ¶P < 0.05 vs. 0.1 μM inhibitor. #P < 0.05 vs. 1.0 μM inhibitor.
lation of downstream targets of p38 (30). Pretreatment with an inhibitor of either MAPK significantly reduced thymidine incorporation. The reduction in thymidine uptake was dependent on the dose of the inhibitor (Fig. 5, B and C). At higher doses (1–10 μM), both SB-203580 and PD-98059 also reduced the basal level of thymidine uptake in control cells not treated with any growth factor. The PKC inhibitors GF-109203X, Gö-6983, and M-PKCI also suppressed mitosis significantly in a dose-dependent manner (Fig. 5, D–F), and a concentration of 0.1 μM of these inhibitors was sufficient to significantly reduce HGF-induced thymidine uptake. Taken together, these results suggest that both p38 and p42/p44 are necessary components of the pathways responsible for mitogenesis induced by HGF in H441 cells. Furthermore, they indicate that PKC is also involved in the mitogenic pathway stimulated by HGF but not through its effects on MAPKS.

DISCUSSION

Recent findings indicate that HGF may be important in the cellular remodeling and epithelial hyperplasia that accompanies chronic lung injury. Increased amounts of HGF are found in the lungs of animals with acute (39) and chronic (33) lung injuries. HGF stimulates the mitosis of type II cells (18). HGF induces branching morphogenesis when added to fetal lung explants (22, 27). In addition, HGF may be involved in the metabolic changes that accompany chronic lung injury. HGF when given to primary cultures of alveolar type II cells inhibits the secretion of surfactant phosphatidylcholine (33). Thus understanding the mechanisms of action of HGF may provide insight into two of the major processes that are ongoing in chronic injury.

In this study, we provide information on the pathways used by HGF to stimulate mitosis, data that may be relevant to the proliferation of the small-airway and alveolar epithelial cells seen in chronic lung injury. HGF stimulates thymidine uptake and activates both p42/p44 MAPK and p38 MAPK. HGF also activates membrane-associated PKC, but it does not use a PKC-dependent pathway in its activation of the MAPK cascade. When inhibitors relatively specific for PKC and p42/p44 and p38 MAPK were used, we found that the stimulation of thymidine uptake by HGF was abolished. These results suggest that activation of all three serine/threonine kinases is necessary for HGF-induced mitogenesis. To evaluate this possibility, we used all inhibitors at varying doses, and for PKC, we duplicated the experiments with multiple types of inhibitors. The responses to varying concentrations of the MAPK inhibitors as well as to the different types of PKC inhibitors were predictably redundant.

Previously, HGF has been shown to phosphorylate p42/p44 MAPK in mouse keratinocytes (3), human melanocytes (10), rat hepatocytes (1), and human lung adenocarcinoma cells (4); its actions on p38 MAPK have not been demonstrated previously. Activation of MAPK kinase kinase such as Raf-1 by growth factors or stress signals sets in motion the cascade of kinases leading to MAPK activation (16, 23). Although the exact mechanisms of activation of these kinases are still not known, several reports (23, 28) have demonstrated that certain isoforms of PKC are capable of activating Raf-1. Here, we show that HGF can activate both the conventional and atypical isoforms of PKC. However, inhibition of PKC by GF-109203X or PMA does not prevent HGF-induced activation of either MAPK. This is in contrast to the observation made by Adachi et al. (1), who found that inhibitors of PKC reduced HGF-induced p42/p44 MAPK activation. Apparently, the pathway(s) used by HGF to activate p42/p44 MAPK activation may differ with the cell type under investigation. PKC, however, may not be completely without effect on MAPK in these cells. Both GF-109203X and overnight treatment with PMA caused a drop in mean basal p42/p44 MAPK phosphorylation, but there was variability in the results and they were not significant. This finding, however, suggests that a GF-109203X-responsive PKC isoform could be involved in the basal level of p42/p44 MAPK activity, perhaps warranting more complete study in future experiments.

Although p42/p44 MAPK is associated with survival mechanisms and is most strongly activated by mitogenic growth factors (5, 6), another member of the MAPK family, p38 MAPK, has been shown to be involved in apoptosis (6). Thus p42/p44 and p38 MAPKs have often been attributed with the seemingly opposing actions of survival and growth arrest, respectively (6, 14, 15, 36); p38 MAPK is not usually activated by growth factors, but it is activated by cellular stresses and inflammatory cytokines (14, 15, 36). In H441 cells, this paradigm is not reproduced because HGF activates both MAPKs, and the suppression of either of the MAPKs results in an inhibition of cell proliferation. Very recently, a paper appeared (24) that reported that both p42/p44 and p38 were required for proliferation of hematopoietic cells. Targets of the actions of MAPKs were not identified, but the investigators speculated that a cooperative action of the two MAPKs might be involved. Our findings are in consonance with these conclusions, suggesting that the interaction between p42/p44 and p38 may be more ubiquitous than previously thought. Such interactions may be key in the determination as to whether a particular population of lung cells in chronic injury enters the pathways of mitosis or, alternatively, apoptosis.

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