Phosphatidylinositol 3-Kinase Activity Is Required for Hepatocyte Growth Factor-induced Mitogenic Signals in Epithelial Cells*

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Phosphatidylinositol (PI) 3-kinase is an important enzyme implicated in growth factor-stimulated intracellular signaling. In this study we have shown that hepatocyte growth factor (HGF) induces a rapid tyrosine phosphorylation of PI 3-kinase and association with HGF receptor/Met in Mv1Lu epithelial cells. Murine mammary carcinoma (SP1) cells, which co-express HGF and HGF receptor/Met, showed sustained phosphorylation of PI 3-kinase. Wortmannin, a potent inhibitor of PI 3-kinase, inhibited HGF-induced PI 3-kinase activity, proliferation of Mv1Lu cells, and spontaneous growth of SP1 cells in a dose-, and time-dependent manner. Transfection of a dominant negative mutant p85 (Ap85) subunit of PI 3-kinase into SP1 cells strongly inhibited HGF-stimulated proliferation and PI 3-kinase activity. However, wortmannin did not influence HGF-induced c-Jun expression. Furthermore, HGF stimulated S6 kinase activity, but its activity was not required for HGF-induced proliferation. Overall, these results suggest that HGF-induced PI 3-kinase activity is important for the mitogenic action of HGF in epithelial cells and further demonstrate that expression of c-Jun is not influenced by inhibition of PI 3-kinase activity.

Hepatocyte growth factor (HGF) is a protein expressed in a variety of cell types and tissues (1–4). HGF is mitogenic, motogenic, and morphogenic for epithelial cells in culture (5–8). All of these responses are believed to be mediated by HGF receptor, identified as the product of the met protooncogene (9). The HGF receptor/Met is a heterodimeric protein of 190 kDa, which consists of a 45-kDa α-subunit linked by disulfide bonds to a 145-kDa β-subunit (10). The β-subunit spans the membrane and shows tyrosine kinase activity (11).

It has been shown that HGF receptor/Met is autophosphorylated on Tyr1334 and Tyr1356 (12) and that substitution of these residues with Phe significantly reduces kinase activity of the HGF receptor/Met (13, 14). Over the past few years, much effort has been focused to identify the tyrosine kinase(s) that are associated with, and/or are phosphorylated by, HGF receptor/Met following stimulation by HGF. In this context, GAP, Src, mitogen-activated protein kinase, phospholipase C-γ, phosphatidylinositol (PI) 3-kinase, and Ras have been identified as being activated by HGF stimulation (15–17). Two phosphorylation sites, Tyr1349 and Tyr1356, located in the carboxyl terminus of HGF receptor/Met, have been identified as docking sites responsible for binding of Src, Shc, Grb2, and PI 3-kinase (15, 16, 18).

PI 3-kinase is one of the key enzymes activated in HGF-induced signal transduction. It catalyzes phosphorylation of PI to PI 3-phosphate, PI 4-phosphate to PI 3,4-bisphosphate, and PI 4,5-bisphosphate (PIP₂) to PI 3,4,5-trisphosphate (PIP₃) (19–21). Recent studies support the involvement of PI 3-kinase in the action of S6 kinase (22), activation of protein kinase C-ζ (PKC-ζ) (23), membrane ruffling (24), and actin polymerization (25). Although a role for PI 3-kinase in mitogenesis has been suggested (26–28), the direct role of PI 3-kinase or its lipid products in mitogenesis remains unclear.

Evidence that PI 3-kinase is associated with HGF receptor/Met (15) suggested that this enzyme may be necessary for the growth-promoting action of HGF. In this study, we investigated the involvement of PI 3-kinase in HGF-induced mitogenic signals in two epithelial derived cell lines. Our results suggest that PI 3-kinase is physically associated with HGF receptor/Met and is activated upon HGF stimulation. Transfection of a dominant negative mutant p85 subunit of PI 3-kinase into murine mammary carcinoma (SP1) cells and treatment of these cells with wortmannin inhibited HGF-induced proliferation and PI 3-kinase activity. Inhibition of PI 3-kinase activity also caused a pronounced reduction in tyrosine phosphorylation of S6 kinase. However, the involvement of PI 3-kinase in HGF-induced mitogenic signaling appears not to be mediated by regulating the expression of the transcription factor, c-Jun.

EXPERIMENTAL PROCEDURES

Materials—ATP, PI, and wortmannin were purchased from Sigma. [γ-32P]ATP, [32P]orthophosphate, and enhanced chemiluminescence (ECL) reagent were purchased from Amersham Corp. Rapamycin was from Calbiochem. Rabbit anti-rat PI 3-kinase IgG (specific for the p85 subunit) and anti-rat/human S6 kinase IgG were purchased from UBI. Mouse anti-phosphotyrosine (PY20) monoclonal antibody was purchased from Transduction Laboratories. Rabbit anti-c-Jun IgG was from Oncogene Science. Rabbit anti-Met IgG was kindly provided by Dr. M. Park (McGill University, Montreal, Canada) (29). Recombinant human HGF was kindly provided by Dr. R. Schwall (Genetech, Inc., San Francisco, CA). Monoclonal anti-bovine p85 antibody (G12) and cDNA encoding a dominant negative mutant p85 into SP1 Cells—The expression vector SRe-Ap85 containing cDNA encoding the mutant bovine p85 (Ap85) subunit, which lacks the binding site for the p110 catalytic subunit of PI 3-kinase, was obtained from M. Kasuga (30). The expression vector pPGKβgeo/pA containing cDNA encoding the neomycin resistance
gene ligated to the β-galactosidase gene was obtained from S. Soriano (33). An eukaryotic plasmid of SP1 cells was cotransfected with S. Soriano's ppgK(C)geo/pβPA expression vectors at a DNA ratio of 2:1 using the LipofectAMINE method (Life Technologies, Inc.). Twenty-four clones were selected in G418 (250 μg/ml, Life Technologies, Inc.) after 3–4 weeks and were tested for proliferation. Four clones were chosen for biochemical analysis.

**Immunoprecipitation and Western Blotting of PI 3-Kinase—**Mv1Lu and SP1 cells were grown to confluency and serum-starved for 24 h. Cells were rinsed with cold phosphate-buffered saline buffer three times and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1% Nonidet P-40, 2 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 μM pepstatin. Lysates were centrifuged for 10 min at 14,000 rpm at an ice bath to remove the nuclear protein fraction. The supernatants were adjusted for protein content using a Micro BCA protein assay (Pierce), and were adjusted to equal protein concentrations. Equal volumes of each supernatant were incubated with polyclonal anti-p85 IgG (5 μg/ml) or monoclonal anti-bovine p85 IgG (G12) (1 μg/ml) at 4 °C for 1 h. Immunoprecipitates were collected on protein A-Sepharose (rabbit anti-mouse IgG was used as a secondary antibody for immunoprecipitation of anti-p85 p85 IgG with protein A-Sepharose), washed three times with lysis buffer, separated on 8% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane. The membrane was blocked with 3% bovine serum albumin and probed with anti-Tyr(P) antibody (1:5000). The membrane was washed four times with TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 1 h, probed with horseradish peroxidase-conjugated secondary donkey anti-rabbit IgG (Amersham) or goat anti-mouse IgG (Cappel) (1:5000) for 1 h, and washed three times with TBST. Immunocomplexes were detected using ECL.

**Cell Proliferation Assay—**Mv1Lu and SP1 cells were plated at 10^5 cells/well in 24-well plates under the various conditions indicated. DNA synthesis was measured at 2 days by adding 0.2 μCi [3H]thymidine ([3H]TdR) (Amersham) at 24 h. After an additional 24 h, cells were harvested with trypsin/EDTA. Aliquots of cells were placed in 96-well microtiter plates and transferred to filters using a Titertek cell harvester (Flow Laboratories), and [3H]thymidine incorporation was measured in a scintillation counter. Results are expressed as the mean cpm/well ± S.D. of triplicate determinations.

**PI 3-Kinase Assay—**PI 3-kinase was assayed as described previously (34, 35). Briefly, PI 3-kinase was immunoprecipitated from SP1 and Mv1Lu cell lysates with anti-Tyr(P) antibody, as described above. PI 3-kinase activity was measured by incubating immunoprecipitates with PI (20 μg), 100 mM MgCl_2, 0.88 mM ATP, [γ-32P]ATP (30 μCi), and a buffer containing 100 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, for 20 min at 30 °C with constant agitation at room temperature. The reaction was terminated by adding 20 μl of 6X HCl, and lipids were extracted by the addition of 160 μl of CHCl_3/CH_3OH (1:1), and were separated in CHCl_3/CH_3OH/H_2O/NH_4OH (60:47:11.3:2) by TLC. The TLC plate was dried and autoradiographed, and the radiolabeled PI 3-phosphate spots were scraped from the plate and counted by liquid scintillation.

**RESULTS**

**HGF Stimulates Tyrosine Phosphorylation of PI 3-Kinase and Association with HGF Receptor/Met—**HGF is capable of activating multiple signal transduction pathways in its target cells (15–17). To examine whether PI 3-kinase participates in HGF-induced signal transduction, we used immunoprecipitation and Western blotting experiments to assess the involvement of this enzyme in HGF-induced signal transduction. Using an anti-PI 3-kinase IgG and anti-Tyr(P) antibody, we first showed that PI 3-kinase is tyrosine-phosphorylated in Mv1Lu cells after treatment with HGF for 20 min. In addition, SP1 cells, which co-express HGF and HGF receptor/Met (8), showed sustained tyrosine-phosphorylated PI 3-kinase (Fig. 1A).

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**FIG. 1.** HGF stimulates tyrosine phosphorylation of PI 3-kinase and association with HGF receptor/Met. A, cell lysates from Mv1Lu cells and SP1 cells untreated (+) or treated (+) with HGF (40 ng/ml) were adjusted to equal protein concentration per group and immunoprecipitated with anti-PI 3-kinase IgG. The precipitates were subjected to 8% SDS-PAGE under reducing conditions, transferred to nitrocellulose membrane, and immunoblotted with anti-Tyr(P) antibody. B, cell lysates from Mv1Lu cells, untreated (+) or treated (+) with HGF (40 ng/ml), were immunoprecipitated with anti-Met IgG, electrophoresed as in A, and subjected to Western blotting with anti-PI 3-kinase IgG. Immunocomplexes were detected by the ECL kit. The arrow indicates the p85 subunit. The protein molecular weight standards are shown on the right.

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measured by adding [3H]thymidine (0.2 μCi/well) at 24 h and incubating for an additional 24 h before cell harvest. Cells were harvested using trypsin/EDTA, transferred onto filter paper using a Titertek cell harvester, and counted in a scintillation counter. The results are expressed as the mean (cpm/well) ± S.D. of triplicate determinations.

**Proliferation**—To determine whether PI 3-kinase was required for HGF-induced proliferation of Mv1Lu cells and SP1 cells, two approaches were used: (a) inhibition of PI 3-kinase with wortmannin, a known inhibitor of PI 3-kinase (36–38), and (b) inhibition of PI 3-kinase activity in SP1 cells by transfection with the cDNA of a dominant negative mutant of the p85 subunit of PI 3-kinase that lacks the binding site for the p110 catalytic subunit of PI 3-kinase. Our initial experiments showed that wortmannin inhibited HGF-induced proliferation of Mv1Lu cells and spontaneous growth of SP1 cells in serum-free medium in a dose-dependent manner (Fig. 2). Among twenty-four clones isolated, four clones were chosen for further study. All four clones responded equally to 10% fetal bovine serum.

**Activation of S6 Kinase Is Not Required for HGF-induced Mitogenic Signals**—The precise mechanism by which PI 3-kinase regulates cell growth is not well defined. Recent studies demonstrate that S6 kinase may act downstream of PI 3-kinase. Treatment of Mv1Lu cells with wortmannin lowered phosphorylation of S6 kinase by 50% (Fig. 6). These results support the notion that S6 kinase acts downstream of PI 3-kinase in this system, in agreement with other reports (22). The inhibition of phosphorylation of S6 kinase is not due to a direct effect of wortmannin on S6 kinase, since wortmannin...
has no effect on the activity of purified S6 kinase (39). To further evaluate whether S6 kinase is required for HGF-induced mitogenic signals, serum-starved SP1 and Mv1Lu cells were treated with rapamycin, a potent inhibitor of S6 kinase (40). Incubation of cells with rapamycin at different concentrations (5–20 ng/ml) did not inhibit HGF-induced cell proliferation in Mv1Lu cells or sustained proliferation of SP1 cells (Fig. 7). HGF-induced Mv1Lu cell proliferation also was not inhibited with 100 ng/ml of rapamycin (data not shown). Rapamycin (50–100 ng/ml) inhibited S6 kinase activity by at least 50% in SP1 and HGF-stimulated Mv1Lu cells (data not shown). Thus, although S6 kinase activity is stimulated with HGF, activation of this enzyme is not required for HGF-induced mitogenic signals. Wortmannin also had no effect on the constitutive phosphorylation of HGF receptor/Met in SP1 cells or on the HGF-induced phosphorylation of HGF receptor/Met in Mv1Lu cells (data not shown). These findings imply that PI 3-kinase activity is not necessary for HGF-induced c-Jun expression.

FIG. 4. Overexpression of a dominant negative mutant of the p85 subunit (Δp85) of PI 3-kinase inhibits HGF-induced mitogenic signals in SP1 cells. Stable transfection of the dominant negative mutant of the bovine p85 (Δp85) into SP1 cells was carried out using the lipofectamine procedure as described under “Experimental Procedures.” Twenty-four clones were selected in G418, and expression of Δp85 was assessed by Western blotting analysis. A, nontransfected SP1 cells (wild type) and four selected clones (C1, C21, C22, and C23) were cultured at 2 × 10^4 cells/well in 24-well plates with 0.1% fetal bovine serum, 0.1% fetal bovine serum plus HGF (40 ng/ml), or 10% fetal bovine serum. DNA synthesis was measured as in Fig. 2. The results are expressed as the mean cpm/well ± S.D. of triplicate determinations. B, cell lysates from wild type nontransfected (W) and selected transfected clones (C1, C21, C22, and C23) were immunoprecipitated with monoclonal anti-bovine p85 antibody. The proteins were transferred to nitrocellulose membrane and immunoblotted with rabbit anti-p85 antibody, which recognizes both mouse and bovine p85. Subsequently, immunocomplexes were detected by the ECL reagent.

FIG. 5. Overexpression of a dominant negative mutant of the p85 (Δp85) subunit of PI 3-kinase inhibits PI 3-kinase activity in SP1 cells. Wild type SP1 cells and four selected clones transfected with a dominant negative mutant of bovine p85 (Δp85) were analyzed for PI 3-kinase activity. Proteins were immunoprecipitated from nontransfected (W) and selected clones (C1, C21, C22, and C23) with anti-Tyr(P) antibody, and kinase activity was measured as described under “Experimental Procedures.” Similar results were obtained following immunoprecipitation with anti-PI 3-kinase IgG. Results are expressed as the percentage of lipid corresponding to 32P-labeled PI 3-phosphate, compared with control values.

DISCUSSION

PI 3-kinase is an important enzyme implicated in growth factor-stimulated intracellular signaling. Recent studies support the involvement of PI 3-kinase in the action of S6 kinase (22), activation of PKC (23), membrane ruffling (24), and actin polymerization (25). Evidence supports the involvement of differential signal transduction pathways in HGF-induced functions. For example, Ras activation by HGF is required for HGF-induced cell motility (42). In addition to Ras, other signaling molecules activated by HGF include phospholipase C-γ and PI 3-kinase (15–17). Phospholipase C-γ catalyzes the hydrolysis of (PIP2), which yields diacylglycerol and inositol 1,4,5-
triphosphate, resulting in the activation of PKC. Activation of PKC negatively regulates HGF receptor/Met kinase activity (43). On the other hand, phosphorylation of PIP2 by PI3-kinase yields PIP3, the function of which has yet not been established.

In the present study we have shown that HGF induces a rapid tyrosine phosphorylation of PI3-kinase and association with HGF receptor/Met in Mv1Lu cells. SP1 cells, which co-express HGF and HGF receptor/Met (8) showed sustained phosphorylation of PI3-kinase. The expression of the dominant negative mutant Δp85 in SP1 cells strongly inhibited HGF-induced cell proliferation. In addition, wortmannin, a potent inhibitor of PI3-kinase, inhibited HGF-induced PI3-kinase activity and proliferation of Mv1Lu cells and spontaneous growth of SP1 cells in a dose- and time-dependent manner. Maximal inhibition of PI3-kinase and proliferation was observed with 40 ng/ml of wortmannin. In addition, wortmannin also inhibited HGF-induced tyrosine phosphorylation of S6 kinase, suggesting that S6 kinase may act downstream of PI3-kinase.

Recent studies support the involvement of S6 kinase in cell growth of some cell types. Microinjection of antibodies that inhibit S6 kinase and the use of rapamycin, which blocks S6 kinase activity, showed that S6 kinase is important for G1/S phase transition in some cells (44). We therefore examined whether S6 kinase stimulation by HGF is required for HGF-induced cell proliferation. Incubation of SP1 cells and Mv1Lu cells with different concentrations of rapamycin did not influence phosphorylation of PI 3-kinase. The expression of the dominant negative mutant Δp85 in SP1 cells strongly inhibited HGF-induced cell proliferation. In addition, wortmannin, a potent inhibitor of PI 3-kinase, inhibited HGF-induced PI 3-kinase activity and proliferation of Mv1Lu cells and spontaneous growth of SP1 cells in a dose- and time-dependent manner. Maximal inhibition of PI 3-kinase and proliferation was observed with 40 ng/ml of wortmannin. In addition, wortmannin also inhibited HGF-induced tyrosine phosphorylation of S6 kinase, suggesting that S6 kinase may act downstream of PI 3-kinase.
ence HGF-induced cell growth. Our results indicate that although S6 kinase is stimulated by HGF, activity of this enzyme is not required for HGF-induced mitogenic signals. To gain insight into the regulatory mechanisms and downstream targets of PI 3-kinase, we therefore evaluated the effect of inhibition of PI 3-kinase on the expression of c-Jun. Our results demonstrate that HGF stimulates a rapid induction of c-Jun protein; however, inhibition of PI 3-kinase does not affect HGF-induced c-Jun expression.

The mechanism by which the HGF receptor/Met stimulates c-Jun expression is not known; however, recent reports indicate that c-Jun expression is regulated in a PKC-dependent manner. 12-O-Tetradecanoylphorbol-13-acetate, a potent activator of PKC (45), and other agents that lead to PKC activation, such as serum and growth factors, also induce expression of c-Jun (46). Inhibitors of PKC block these induction responses (46, 47). PKC is also activated in response to HGF (48); it is therefore possible that HGF-induced c-Jun expression is regulated by stimulation of PKC by HGF receptor/Met, and thus PI 3-kinase activity may not be required for induction of this gene.

Overall, these results suggest that HGF-induced PI 3-kinase activity is important for the mitogenic action of HGF in epithelial cells and further demonstrate that expression of c-Jun is not influenced by inhibition of PI 3-kinase activity. During the preparation of this work, Royal and Park (49) also reported that PI 3-kinase activity is required for HGF-induced scatter activity of Madin-Darby canine kidney cells. Collectively, these results suggest that PI 3-kinase plays a critical role in many HGF-induced cellular functions.

Taken together, our results indicate that PI 3-kinase is activated in response to HGF and is associated with HGF receptor/Met, and they further suggest that PI 3-kinase plays a major role in HGF-induced cell proliferation. However, the involvement of PI 3-kinase in HGF-induced mitogenic signaling appears not to be mediated by regulating the expression of the transcription factor, c-Jun. Further work will determine whether PI 3-kinase-independent pathways, e.g. PKC that stimulates c-Jun expression (45–47), are also involved in HGF-stimulated proliferation.

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