The receptor for hepatocyte growth factor, also known as scatter factor (HGF/SF), has recently been identified as the 190-kDa heterodimeric tyrosine kinase encoded by the MET proto-oncogene (p190\text{MET}). The signaling pathway(s) triggered by HGF/SF are unknown. In A549 cells, a lung epithelial cell line, nanomolar concentrations of HGF/SF induced tyrosine phosphorylation of the p190\text{MET} receptor. The autophosphorylated receptor coprecipitated with phosphatidylinositol 3-kinase (PI 3-kinase) activity. In GTL16 cells, a cell line derived from a gastric carcinoma, the p190\text{MET} receptor, overexpressed and constitutively phosphorylated on tyrosine, coprecipitated with PI 3-kinase activity and with the 85-kDa PI 3-kinase subunit. In these cells activation of protein kinase C or the transduction of signals generated by tyrosine kinase receptors includes generation of D-3-phosphorylated inositol phospholipids.

Hepatocyte growth factor was isolated as a powerful mitogen for hepatocytes and other epithelial cells (1-3). Scatter factor was independently isolated as a chemotactic factor promoting epithelial cell motility and tumor invasion (4, 5). Recent work has shown that the two factors are identical proteins encoded by the same gene (6, 7). The different biological effects of hepatocyte growth factor/scatter factor (HGF/SF) are triggered by binding to the same receptor, the protein encoded by the MET proto-oncogene (7). The Met protein had already been identified as the receptor for hepatocyte growth factor (8, 9).

The MET-encoded receptor is a 190-kDa heterodimeric transmembrane protein (p190\text{MET}) made of a 50-kDa α subunit disulfide-linked to a 145-kDa β subunit (p145\text{β}) (10, 11). The α subunit and the NH-terminal portion of p145\text{β} are exposed at the cell surface (12). The carboxyl-terminal portion is cytoplasmic and includes a tyrosine kinase domain (13-15), and phosphorylation sites are involved in the regulation of its activity (16). The kinase activity is positively regulated by autophosphorylation on tyrosine (17), and it is negatively regulated by protein kinase C activation (18) or a transient increase of intracellular Ca\textsuperscript{2+} concentration (19). Stimulation of p145\text{β} subunit tyrosine phosphorylation after exposure to HGF/SF was observed both in intact cells (7-9) and in vitro with partially purified Met protein (9).

The signaling pathway(s) triggered by HGF/SF have not yet been investigated. Similarly, the substrates of the p190\text{MET} receptor kinase are unknown. In this work we show that the tyrosine-phosphorylated form of p190\text{MET} receptor associates with phosphatidylinositol 3-kinase, a key enzyme involved in the transduction of signals generated by tyrosine kinase receptors (20, 21).

EXPERIMENTAL PROCEDURES

Reagents, Cell Lines, and Antibodies—All reagents, unless specified, were purchased from Sigma. Protein A covalently coupled to Sepharose was purchased from Pharmacia LKB Biotechnology Inc. Reagents for SDS-PAGE were from Bio-Rad. 3\textsuperscript{H}-Methylated molecular mass standards were from Amersham Corp.

A549 lung carcinoma cells were obtained from American Type Cell Culture. GTL16 is a clonal cell line derived from a poorly differentiated gastric carcinoma (12). Cells were cultured in RPMI 1640 medium (Seromed) containing 10% fetal calf serum (Seromed) and maintained at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. HGF/SF was purified from the supernatant of MRC5 human fibroblasts by heparin-Sepharose column chromatography (Phar- macia) eluted by a linear gradient of 0.5-1.8 M NaCl as described (6).

Phosphotyrosine antibodies were raised as previously described (22). Monoclonal antibodies against the anti-epidermal growth factor receptor antibodies were raised as described elsewhere (48). Polyclonal antibodies against the 85-kDa subunit of PI 3-kinase were raised in chicken immunized with a synthetic peptide as described elsewhere. Preparation of Cell Lysates, Immunoprecipitation, and Western Blotting—Subconfluent A549 cells were starved for 2 days in serum-free medium, stimulated by HGF/SF as indicated, and then lysed in buffer A (25 mM Hepes buffer, pH 8, 10% glycerol, 1% Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 2 mM EGTA, 0.5 mM dithiothreitol, 0.2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HPLC, high pressure liquid chromatography; TPA, phorbol 12,13-dibutyrate).

\textsuperscript{1} The abbreviations used are: HGF/SF, hepatocyte growth factor/scatter factor; PI 3-kinase, phosphatidylinositol 3-kinase; PI 4-kinase, phosphatidylinositol 4-kinase; PI, phosphatidylinositol; SIP, PI(3)P, PI(4)P, PI(3,4)P\textsubscript{2}, PI(3,4,5)P\textsubscript{3}; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; HPCL, high pressure liquid chromatography; TPA, 12-0-tetradecanoylphorbol-13-acetate.
phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1 µg/ml soybean trypsin inhibitors) supplemented with 0.5 mM sodium vanadate, 0.1 mM ammonium molybdate, 10 µM phenylarsine oxide, and 1 mM ZnCl₂. Subconfluent monolayers of GTL16 cells were serum-starved for 2 days and, unless otherwise indicated, lysed in buffer A containing 0.1 mM vanadate. Lysates were spun at 15,000 x g for 15 min, and the supernatants were immunoprecipitated after 2 h of incubation with monomeric anti-Met antibodies cross-linked to rabbit anti-mouse Ig-Protein A-Sepharose. The immunoprecipitates were washed as previously described (23). Western blotting from both whole cell lysate or immunoprecipitates was performed as described before (17, 19).

PI 3-Kinase Assay—PI 3-kinase assay was performed directly on the beads as described by Auger et al. (23). Adenosine (0.2 mM) was added to the reaction mixture to inhibit residual PI(4) kinase activity (33). For definitive identification,PIP, PIP₂, and PIP₃ separated by TLC were eluted, deacylated, and the respective glycerophosphoinositol phosphate derivatives separated on HPLC by a Partisil SAX column (Whatman) as described (23, 24).

Binding of PI 3-Kinase to p19⁰MET in Vitro—Confluent GTL16 cells were lysed in buffer A, and p19⁰MET was purified by immunoprecipitation as described above. The Protein A-Sepharose-immobilized p19⁰MET was dephosphorylated by alkaline phosphatase (Promega) treatment for 20 min at 15 °C. The immunocomplexes were washed as in the immunoprecipitation protocol. When indicated samples were rephosphorylated by incubation for 15 min at room temperature with 0.1 mM ATP, 10 mM MnCl₂. The immunocomplexes were extensively washed as above. A549 cells were made quiescent by 48-h serum starvation and lysed with buffer A supplemented with 0.5 mM sodium vanadate, 0.1 mM ammonium molybdate, and 1 mM ZnCl₂. The lysates were then incubated for 1 h at 4 °C with the immobilized p19⁰MET. The complexes were washed as described in the immunoprecipitation protocol and probed as indicated.

RESULTS

HGF/SF Induces Tyrosine Phosphorylation of p19⁰MET Receptor and Association with PI 3-Kinase in A549 Cells—Stimulation of A549 cells for 15 min with 50 ng/ml purified HGF/SF induced tyrosine phosphorylation of the p19⁰MET receptor. This was observed by probing the anti-Met immunoprecipitates with anti-phosphotyrosine antibodies. The p145⁰ subunit was heavily phosphorylated on tyrosine after 15 min of stimulation with HGF/SF (Fig. 1, A and B). Notably, two additional proteins of molecular mass close to 85 and 60 kDa were also co-precipitated by anti-MET antibodies and were found to contain phosphotyrosine. 85 kDa is the molecular mass of the putative regulatory subunit of PI 3-kinase (26–28, 37), which was found to be phosphorylated on tyrosine when present in a complex with several tyrosine-phosphorylated proteins (reviewed in 20). PI 3-kinase activity, assayed in the presence of adenosine to inhibit residual non-specifically bound PI 4-kinase (33), was present in the immunocomplexes from HGF/SF-stimulated cells. Fig. 1C shows the TLC separation of the d-3-phosphorylated inositol lipids generated in vitro by incubation of the anti-Met immunocomplex in the presence of sonicated lipid substrates and [γ-³²P] ATP. The identities of PIP, PIP₂, and PIP₃ generated in vitro were confirmed by HPLC analysis of the deacylated derivatives to be PI(3)P, PI(3,4)P₂, and PI(3,4,5)P₃ (not shown). No PI 3-kinase activity was detectable in immunocomplexes precipitated from unstimulated cells.

Tyrosine Phosphorylation of p19⁰MET Receptor Regulates the Physical Association with PI 3-Kinase in Intact GTL16 Cells—In GTL16 cells, due to the amplification of the MET gene, the p19⁰MET receptor is overexpressed and constitutively phosphorylated on tyrosine (10). Moreover, we have previously shown that in these cells it is possible to negatively regulate the kinase activity of p19⁰MET by activation of protein kinase C or by increasing the intracellular Ca²⁺ concentration (18, 19). We therefore took advantage of this model system to further investigate if tyrosine phosphorylation of p19⁰MET regulates its association with PI 3-kinase. Subconfluent cultures of GTL16 cells were starved for 3 days in serum-free medium and treated either with TPA (160 nM for 1 h) or with the Ca²⁺ ionophore A23187 for 5 min. p19⁰MET tyrosine phosphorylation was assessed by probing the blotted whole cell lysates with anti-phosphotyrosine and with anti-

Fig. 1. HGF/SF triggers tyrosine phosphorylation of the p145⁰ receptor subunit and promotes its association with PI 3-kinase. Control (–) or HGF/SF-stimulated (+) A549 cells were lysed and immunoprecipitated with anti-Met antibodies. Proteins in the immunocomplexes were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-phosphotyrosine antibodies (panel A) or anti-Met antibodies (panel B). The immunocomplexes were assayed for PI 3-kinase activity in the presence of a mixture of PI, P(4)P, P(4,5)P₂, phosphatidylinositol, and [γ-³²P]ATP. Panel C shows the autoradiogram of a thin layer chromatography separation of the reaction products extracted by chloroform/methanol. The migration of PIP, PIP₂, and PIP₃ is indicated by arrows. The radioactivity at the origin (ORI) is due to [γ-³²P]ATP and other water-soluble ³²P-labeled materials that were not completely extracted from the chloroform layer.

Fig. 2. Tyrosine phosphorylation of the HGF/SF receptor p145⁰ subunit is negatively regulated by protein kinase C activation and increase of intracellular Ca²⁺. Proteins solubilized from GTL16 cells were separated in SDS-PAGE transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies (panel A) or anti-Met antibodies (panel B). Lane 1, control cells; lane 2, cells treated with 160 nM TPA for 1 h; lane 3, cells treated with the 10 µM Ca²⁺ ionophore A23187 for 5 min.
Met antibodies (Fig. 2). The p190\textsuperscript{MET} receptor was then immunoprecipitated from control and TPA or A23187 Ca\textsuperscript{2+} ionophore-treated cells; immunoprecipitates were blotted and probed with either anti-phosphotyrosine or anti-Met antibodies. The amount of p190\textsuperscript{MET} tyrosine phosphorylated in the immunoprecipitates from untreated cells was severalfold higher (Fig. 3, A and B). The PI 3-kinase activity, measured as Δ-3-phosphoinositides generated \textit{in vitro}, co-precipitated with tyrosine phosphorylated p190\textsuperscript{MET} from control cells but could not be detected in the immunocomplexes from TPA and Ca\textsuperscript{2+} ionophore-treated cells (Fig. 3D).

To demonstrate the physical association of the 85-kDa subunit of PI 3-kinase with tyrosine-phosphorylated p190\textsuperscript{MET}, immunoprecipitates from GTL16 cells, either control or treated with TPA or the Ca\textsuperscript{2+} ionophore, were blotted and probed with chicken antibodies directed against the 85-kDa subunit of PI 3-kinase with tyrosine-phosphorylated p190\textsuperscript{MET} from control cells but could not be detected in the immunocomplexes from TPA and Ca\textsuperscript{2+} ionophore-treated cells (Fig. 3D).

**Tyrosine Phosphorylation of p190\textsuperscript{MET} Receptor Is Required for Binding of PI 3-Kinase in Vitro**—In order to formally prove that p190\textsuperscript{MET} tyrosine phosphorylation is a switch for association of PI 3-kinase, we developed an \textit{in vitro} association assay. Sepharose-immobilized p190\textsuperscript{MET} receptors were dephosphorylated by alkaline phosphatase treatment and extensively washed to remove any associated PI 3-kinase activity. In half of the samples the receptors were re-phosphorylated on tyrosine as described under “Experimental Procedures.” The unphosphorylated and the re-phosphorylated immobilized receptors were incubated for 1 h at 4 °C with a lysate prepared from A549 cells or with the lysis buffer alone. The cell lysates did not contain any anti-phosphotyrosine immunoprecipitable PI 3-kinase activity, suggesting that the enzyme neither is phosphorylated on tyrosine nor is associated with tyrosine-phosphorylated proteins (data not shown). However, PI 3-kinase activity from the cell lysate did bind to the tyrosine-phosphorylated p190\textsuperscript{MET} receptor. No PI 3-kinase activity associated with the unphosphorylated receptor, and no residual activity was found in samples incubated with buffer alone (Fig. 4C). The amount of p190\textsuperscript{MET} bound to the beads and its state of phosphorylation were not affected by the incubation with A549 cell lysate; as shown by probing with anti-Met and anti- phosphotyrosine antibodies the immunocomplexes incubated with either cell lysates or buffer alone (Fig. 4, A and B). This experiment demonstrates that tyrosine phosphorylation of p190\textsuperscript{MET} is a switch for association with PI 3-kinase in vitro.

**DISCUSSION**

In this paper we present the first evidence that tyrosine phosphorylation of the HGF/SF receptor is a switch for association of PI 3-kinase. This enzyme plays a key role in the signal transduction pathway by generating Δ-3-phosphoinositides (23, 24), a novel class of putative intracellular second messengers (20, 26). The role in signal transduction of PI(3)P, PI(3,4)P\textsubscript{2}, and PI(3,4,5)P\textsubscript{3} is not yet understood, although their cellular levels \textit{in vivo} are elevated in cells entering the mitotic cycle or transformed by activated oncogenes (23, 34–36). PI 3-kinase, recently purified to homogeneity, is a het-
peptides containing the homologous Tyr739 in the phosphorylated state (39). This tyrosine is included in a sequence highly conserved in other receptor kinases which associate with PI 3-kinase play a role in the signaling pathway (31). These molecules, acting as a regulatory subunit for the catalytic 110-kDa subunit of PI 3-kinase may be regulated by tyrosine phosphorylation (26, 27).

So far PI 3-kinase has been found to associate with and to be activated by a number of tyrosine kinase receptors, including the receptors for PDGF (33), colony-stimulating factor-1 (40), insulin (41, 42), epidermal growth factor (reviewed in 20), and stem cell growth factor (44). The association is ligand-dependent and mediated by tyrosine phosphorylation of the receptor cytoplasmic domain.

It has been shown that phosphorylation of a specific tyrosine (Tyr845) in the kinase insert domain of the human β-PDGFR receptor is critical for association with PI 3-kinase (45). Similarly, in vitro binding of PI 3-kinase to the mouse β-PDGFR receptor is inhibited by competition with synthetic peptides containing the homologous Tyr845 in the phosphorylated state (39). This tyrosine is included in a sequence highly conserved in other receptor kinases which associate with PI 3-kinase. From these homologous sequences a putative consensus (YXXM-) has been deduced (20).

We have shown that PI 3-kinase associates with the p190MT HGF/SF receptor. The association is mediated by tyrosine phosphorylation of the p145 subunit. The intracellular domain of p145 is itself a tyrosine kinase, whose activity is controlled by binding to the specific ligand. P145 also contains multiple tyrosine autophosphorylation sites (16) and a consensus sequence for binding to PI 3-kinase (16). It has been suggested that the recruitment of PI 3-kinase to tyrosine-phosphorylated receptors is a necessary step for activation in vivo (20). Furthermore the 85-kDa subunit of PI 3-kinase was found to be phosphorylated on tyrosine only when associated to the membrane (31). These molecules, acting as a regulatory subunit for the catalytic 110-kDa subunit of PI 3-kinase may be regulated by tyrosine phosphorylation (26, 27).

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