Hepatocyte Growth Factor-induced Differential Activation of Phospholipase Cγ1 and Phosphatidylinositol 3-Kinase Is Regulated by Tyrosine Phosphatase SHP-1 in Astrocytes*

Hepatocyte growth factor (HGF) elicits pleiotropic effects on various types of cells through the c-Met receptor tyrosine kinase. However, the mechanisms underlying the diverse responses of cells remain unknown. We show here that HGF promoted chemokinosis of rat primary astrocytes through the activation of phosphatidylinositol 3 (PI3)-kinase without any influence on mitogenesis of the cells. Under the same condition, phospholipase Cγ1 (PLCγ1), which is another signal mediator of c-Met, was not tyrosine-phosphorylated during HGF stimulation. However, treatment of the cells with orthovanadate, a tyrosine phosphatase inhibitor, restored the HGF-induced tyrosine phosphorylation of PLCγ1. A tyrosine phosphatase, SHP-1, was associated with both PI3-kinase and PLCγ1 before HGF stimulation, but it was dissociated only from PI3-kinase after the stimulation. Furthermore, transfectants of catalytically inactive mutant of SHP-1 showed tyrosine phosphorylation of PLCγ1 and mitogenic responses to HGF, and the mitogenic response was blocked with U73122, an inhibitor of phosphatidylinositol-specific PLC, and calphostin C, an inhibitor of protein kinase C downstream of the PLCγ1. These results indicate that PLCγ1 is selectively prevented from being a signal mediator by constitutive association of SHP-1, and that this selective inhibition of PLCγ1 may determine the cellular response of astrocytes to HGF.

* This work was supported by grants from the Japanese Ministry of Health and Welfare and the Science and Technology Agency of Japan, and by a grant-in-aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, Sports and Culture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: HGF, hepatocyte growth factor; PLC, phospholipase C; PI, phosphatidylinositol; BrdUrd, bromodeoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; GFAP, glial fibrillary acidic protein.

It has been revealed that PI3-kinase is involved in HGF-induced migration of IMCD cells (7) and Madin-Darby canine kidney cells (8), and that PLCγ1 mediates an intracellular signal for the HGF-enhanced mitogenesis of rat primary hepatocytes (9). Furthermore, PLCγ1 is considered to participate in cell migration, since activation of protein kinase C, the downstream effector of PLCγ1, mimics HGF-induced membrane ruffling in KB cells (10) and R308 cells (11).

In addition to the biological significance of HGF in peripheral organs and cells, various effects of HGF on cells of the central nervous system have also been reported (12–15). We have previously found that HGF promotes neurite outgrowth of cultured rat embryonic neocortical explants (13). In this system, tyrosine phosphorylation of PLCγ1 was critical for the neurite outgrowth, whereas PI3-kinase was not phosphorylated during the stimulation (16). These findings suggested that two major downstream effectors of c-Met, PI3-kinase, and PLCγ1 were not necessarily co-activated, but rather differentially regulated in neuronal cells.

In the present study, we analyzed the activation of PI3-kinase and PLCγ1 by HGF stimulation in rat primary astrocytes, a species of glial cells of the central nervous system. We found that HGF specifically stimulated tyrosine phosphorylation of PI3-kinase, not PLCγ1 in the cells. Furthermore, HGF caused rapid dissociation of a tyrosine phosphatase, SHP-1, in a mammalian homologue of Drosophila Csw (17), from PI3-kinase, while the phosphatase still bound to PLCγ1, which may be a biochemical mechanism accounting for the selective activation of PI3-kinase with HGF. Furthermore, PLCγ1 was phosphorylated with tyrosine in the cells expressing a dominant negative mutant of SHP-1, and the cells showed mitogenic responses to HGF. Our study revealed that SHP-1 plays an important role in both selective activation of PI3-kinase and prevention of phosphorylation of PLCγ1 during the stimulation with HGF, and contributes to induction of the novel neurotrophic functions of HGF to the glial cells.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human HGF and monoclonal anti-bromodeoxyuridine (BrdUrd) antibody were obtained from Becton Dickinson. Texas Red-labeled phalloidin was from Molecular Probes. Anti-phosphotyrosine (anti-Tyr(P)) antibody 4G10 was from Seikagaku Kogyo Co., Ltd. Monoclonal anti-PLCγ1 antibody was from Transduction Laboratories. Polyclonal antibody to the p85 subunit of PI3-kinase (anti-PI3-kinase) was from Upstate Biotechnology. Polyclonal antibodies to c-Met, SHP-1, and SHP-2 were from Santa Cruz Biotechnology. BrdUrd, bromodeoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; GFAP, glial fibrillary acidic protein.
Probe. Unless otherwise provided, other reagents were purchased from Sigma.

Cell Culture—Astrocytes were prepared from postnatal 0-day-old Wistar rats by the method reported previously (18). More than 98% of the cells were positive for GFAP staining. The cells were cultured on poly-L-lysine-coated plastic culture dishes at a density of 1 × 10^4/cm^2. The cells were grown to semiconfluence and starved in serum-free Dulbecco's modified Eagle's medium (DMEM) for 10 h.

Analysis of Mitogenic Response of Primary Astrocytes to HGF—The serum-starved cells were unstimulated or stimulated with 0.5 nM HGF or 10% serum, and labeled with 10 μM BrdUrd for 2 h before stimulation or 3 or 5 h after stimulation. The cells were fixed in 4% paraformaldehyde and permeabilized with 70% ethanol. The samples were further treated with 2% HCl for 15 min and neutralized with 0.1 M sodium tetraborate for 30 min. The cells were treated by double staining with anti-GFAP and anti-BrdUrd antibodies, and respectively probed by fluorescein isothiocyanate-labeled anti-mouse immunoglobulin and Texas Red-labeled anti-rabbit immunoglobulin. The number of the cells stained positively for both BrdUrd and GFAP was counted.

Immunoprecipitation and Western Blotting—Immunoprecipitates were determined according to the methods reported previously (26). The immunoprecipitates were subjected to Western blotting with anti-PLCγ1 antibody and anti-Flag antibodies. The same experiments were performed in the presence of 0.5 μM U73122, 50 μM wortmannin, or 50 μM calphostin C.

RESULTS

Tyrosine Phosphorylation of c-Met in Primary Astrocytes during Stimulation with HGF—To explore the function of HGF in astrocytes, we examined first the tyrosine phosphorylation of c-Met during HGF stimulation. Serum-starved astrocytes were stimulated with various concentrations of HGF for 5 min (Fig. 1A) or with 0.5 nM HGF for the indicated periods of time (Fig. 1B). The cells were subjected to immunoprecipitation for c-Met, which was followed by Western blotting with anti-Tyr(P) antibodies. c-Met β-chain (145 kDa) was phosphorylated with tyrosine after stimulation with 0.1–10 nM HGF (Fig. 1A). In the time-course experiment, c-Met was highly tyrosine-phosphorylated within 2 min, and thereafter the phosphorylation level...
Actin reorganization and chemokinesis, not mitogenic response, were promoted by HGF stimulation. A, serum-starved astrocytes were labeled with BrdUrd for 2 h before stimulation, and for the last 2 h of the 5- or 7-h stimulation with 0.5 nM HGF or 10% serum (serum (-), HGF, and 10% serum, respectively). The number of the cells double-stained with anti-GFAP and anti-BrdUrd antibodies was counted. B, serum-starved astrocytes were unstimulated or stimulated with 0.5 nM HGF for 30 min in the presence and absence of 50 nM wortmannin (wort). The actin structures stained with Texas Red-labeled phalloidin are shown. C, serum-starved astrocytes were stimulated with 0.5 nM HGF for the indicated periods of time in the medium with or without 50 nM wortmannin. The cytosolic fractions were immunoblotted with anti-actin antibodies (lower panel). The bands were analyzed by a densitometer, and the results are shown in the upper panel. D, HGF was added to the lower compartment (shown as L) of the chemotaxis chamber separated with a mesh filter with pore size of 5 μm, and the cells were seeded on the upper compartments. The number of the cells on the lower sides of the filter was counted 4 h after the stimulation. The same experiments were performed in the presence of 50 nM wortmannin (wort), 0.5 μM U73122, or the same concentration of HGF in the upper compartment as that in the lower compartment (shown as U/L). The data in A, C, and D are expressed as means ± S.E. of four experiments.

was sustained (Fig. 1B). When the same immunoblots were reprobed with anti-c-Met antibodies, c-Met showed the almost equal recovery in each maneuver of immunoprecipitation. These observations indicated that functional c-Met was expressed in astrocytes.

HGF Promotes Actin Reorganization and Chemokinetic Migration, and Not Mitogenesis of Astrocytes—Since HGF promotes proliferation of various types of cells, we examined effects of HGF on the cell growth of astrocytes. Astrocytes were pulse-labeled with BrdUrd for 2 h, and the number of the cells positive for both BrdUrd- and GFAP-immunostainings was counted. As shown in Fig. 2A, with 0.5 nM HGF had no influence on any mitogenic response, while BrdUrd-positive cells increased by 7 h of incubation with the medium containing 10% fetal bovine serum.

Based on the reports showing that various types of cells exhibit morphological changes or motile responses to HGF, which accompany cytoskeletal reorganization (1, 2), changes in the actin structures were then examined by staining with Texas Red-labeled phalloidin (Fig. 2B). Evident stress fiber-like structures (F-actin) were finely observed in most unstimulated cells; however, the structures disappeared 30 min after HGF stimulation. Simultaneously with the disappearance of the actin cytoskeletal structures, the amount of actin in the soluble cytosolic fraction (G-actin) was increased by the stimulation (Fig. 2C), suggesting that HGF causes conversion of F-actin into G-actin. These morphological and biochemical changes were suppressed by 50 nM wortmannin, an inhibitor of PI3-kinase (Fig. 2, B and C), or 50 μM LY294002, an inhibitor of PI3-kinase, which was structurally unrelated to wortmannin (data not shown), suggesting that the actin reorganization is enhanced by the activation of PI3-kinase.

The HGF-induced changes in actin structures were reminiscent of cell migration. These profiles of migration were assessed by a modified chemotaxis chamber (Fig. 2D). When HGF was supplied to the lower compartment of the chamber, cells that migrated from the upper to the lower side were significantly increased. Then, the cell migration was evaluated by the addition of the same concentration of HGF to both upper and lower compartments. This treatment also increased the number of the cells that migrated toward the lower compartment, indicating that HGF did not function as a chemoattractant but accelerated the cell migration toward random directions (chemokinesis). This HGF-induced motile response was inhibited with 50 nM wortmannin. It has been suggested that PLCγ1 is a potential signal mediator for HGF-promoted cell migration (10, 11); however, U73122, an inhibitor of phosphatidylinositol-specific PLC did not affect the migration of astrocytes. These results suggest that the chemokinetic response of astrocytes to HGF is induced by the activation of PI3-kinase but not PLC.

HGF Activates PI3-kinase in Astrocytes—It has been shown that tyrosine phosphorylation of the 85-kDa subunit of PI3-kinase is an initial step for the recruitment of PI3-kinase into the signal cascades mediated by tyrosine kinase receptors. We therefore monitored tyrosine phosphorylation of the p85 subunit of PI3-kinase during the stimulation of astrocytes with
HGF. As shown in Fig. 3A, tyrosine phosphorylation of PI3-kinase was detected within 2 min and it was gradually enhanced until 10 min after the stimulation with 0.5 nM HGF. Furthermore, several tyrosine-phosphorylated proteins were co-immunoprecipitated with PI3-kinase from the stimulated cells. Then, the catalytic activity of PI3-kinase in tyrosine-phosphorylated proteins was determined in vitro (Fig. 3B). The generation of phosphatidylinositol 3-monophosphate, a product of PI3-kinase, was readily detected from the phosphoproteins immunoprecipitated with anti-Tyr(P) antibody after 2 min stimulation, and the level of phosphatidylinositol 3-monophosphate was increased up to 10 min after the stimulation. These observations were in agreement with the enhanced phosphorylation of the 85-kDa subunit. The measurement performed in the presence of 50 nM wortmannin revealed efficient suppression of the activity. These results demonstrated that PI3-kinase is involved in intracellular signaling of HGF in astrocytes.

Tyrosine Phosphorylation of PLCγ1 Is Suppressed during HGF Stimulation—Second, the involvement of PLCγ1 in c-Met-mediated signal cascades was investigated. Although PLCγ1 was expressed in astrocytes, its tyrosine phosphorylation was never detected following HGF stimulation (Fig. 4A). However, when the cells were pretreated with orthovanadate, an inhibitor of tyrosine phosphatases, PLCγ1 was significantly phosphorylated by HGF stimulation (Fig. 4B). These results suggest that PLCγ1 is a potential signal mediator of c-Met, although its phosphorylation was suppressed by tyrosine phosphatase(s) in astrocytes.

Dissociation of SHP-1 from PI3-kinase and Its Stable Association with PLCγ1 during HGF Stimulation—To investigate the involvement of tyrosine phosphatase(s) in the suppression of tyrosine phosphorylation of PLCγ1, we examined the association of phosphatases with PLCγ1 by in-gel tyrosine phosphatase assays. The tyrosine phosphatase activities of the whole lysates from stimulated and unstimulated astrocytes could be detected as bands at molecular mass units of 50, 65, and 120 kDa (Fig. 5A). When the same analysis was carried out by using anti-PLCγ1 immunoprecipitates, only the 65-kDa phosphatase was detected (Fig. 5B). This phosphatase was constitutively associated with PLCγ1 regardless of HGF stimulation. In addition, the 65-kDa tyrosine phosphatase was associated with PI3-kinase in unstimulated cells. However, the tyrosine phosphatase was clearly dissociated from PI3-kinase after the stimulation.

In consideration of the molecular weight of the tyrosine phosphatase and the binding abilities to PLCγ1 and PI3-kinase (27–32), SHP-1 and the related molecule, SHP-2, are regarded as possible candidates for the association with PLCγ1 and PI3-kinase. The possibility was assessed by blotting the immunoprecipitates with anti-PLCγ1 or PI3-kinase antibodies were prepared from the cells stimulated with or without 0.5 nM HGF for 2 min, and subjected to in-gel tyrosine phosphatase assays. Arrows on the right indicate the bands corresponding to the phosphatase activities.
ated in the cells transfected by inactive SHP-1 even before HGF stimulation, and the phosphorylation was further enhanced by 5 min of HGF stimulation (Fig. 7A). However, transfection of the control vector and wild-type SHP-1 did not enhance the phosphorylation of PLC\(\gamma\) before and after the stimulation by HGF. Likewise, transfection of the Cys\(\rightarrow\)Ser mutant of SHP-2 did not induce tyrosine phosphorylation of PLC\(\gamma\) (Fig. 7A), despite the mutant protein having been abundantly expressed as compared with the mutant of SHP-1 (Fig. 7B). These results suggest that SHP-1 is a critical phosphatase suppressing the phosphorylation of PLC\(\gamma\) following HGF stimulation.

**SHP-1 Prevents the PLC\(\gamma\)i-mediated Mitogenic Response to HGF**—Based on the report showing that activation of PLC\(\gamma\) is required for epidermal growth factor- and platelet-derived growth factor-promoted cell proliferation (33), prevention of tyrosine phosphorylation of PLC\(\gamma\) by SHP-1 may be attributed to lack of mitogenic response of astrocytes to HGF. Accordingly, we examined the possibility of the transfectants of mutant SHP-1 being used (Fig. 8). HGF did not enhance the proliferation of astrocytes which were transfected with the control vector or wild-type SHP-1, as in the case shown in Fig. 2A. However, the mutant SHP-1-transfectants exposed to HGF exhibited the increasing mitogenic response after the stimulation (Fig. 8A). The effect of mutant SHP-1 was inhibited with suboptimum concentration (0.5 \(\mu\)M) of U73122, an inhibitor of phosphatidylinositol-specific PLC and 50 nM wortmannin, indicating that simultaneous activation of PLC\(\gamma\) and PI3-kinase is essential for the mitogenic response to HGF in astrocytes (Fig. 8A). Furthermore, calphostin C, an inhibitor of diacylglycerol-specific protein kinase C (PKC), which is a downstream effector of PLC\(\gamma\), also inhibited the mitogenic response emerged by mutant SHP-1 (Fig. 8B). These results strongly suggest that PLC\(\gamma\)i is a potential signal mediator for HGF-induced cell proliferation, which was constitutively suppressed by SHP-1 in astrocyte.

**DISCUSSION**

This article describes how HGF promotes actin reorganization and chemokineti migration of astrocytes (Fig. 2), which is the first demonstration of the effects of HGF on glial cells as well as neuronal cells of the central nervous system. Promotion of cell motility is a typical activity of HGF observed in various types of cells of peripheral organs (1). With regard to the intracellular signaling underlying the biological effect, PLC\(\gamma\) and its downstream effector(s), PKC(s) (10, 11, 34), and PI3-
kinase (7, 8) have been reported to function as mediators in this process. However, it has not been clarified whether the activation of both PLCγ1 and PI3-kinase is necessary for HGF-induced cell migration. Our results clearly indicated that HGF selectively induced tyrosine phosphorylation and activation of PI3-kinase and not PLCγ1 in astrocytes, and that the activation of PI3-kinase was essential for the HGF-induced cell migration. This selective activation of PI3-kinase by HGF is unique to astrocytes, since both PI3-kinase and PLCγ1 are activated in most of the cell types (6, 9, 34) or only PLCγ1 is activated by HGF in neurons (16). These results suggest that the selective activation of PI3-kinase determines the cellular response of astrocytes to HGF.

PLCγ1 is also an essential signal mediator for the mitogenesis promoted by various growth factors (33). Through the experiment, tyrosine phosphorylation of PLCγ1 or mitogenic response could not be detected in astrocytes stimulated with HGF (Figs. 2A and 4A). In the cells treated with NaN₃VO₄, an inhibitor of protein-tyrosine phosphatases, however, PLCγ1 was significantly tyrosine-phosphorylated at as other types of cells (Fig. 4B). This observation led us to identify SHP-1 as a PLCγ1-associated protein-tyrosine phosphatase (Figs. 5 and 6A). The association was sustained during HGF stimulation up to 10 min of examination (data not shown). Furthermore, transfection of a catalytically inactive mutant of SHP-1 resulted in tyrosine phosphorylation of PLCγ1 by HGF (Fig. 7), and the cells showed a mitogenic response to HGF (Fig. 8). This mitogenic response was inhibited by an inhibitor of PLC. The inhibitor could not suppress astrocytic migration stimulated by HGF (Fig. 2D), indicating that the cells were viable under the treatment. These results indicated that PLCγ1 was prevented from being activated by SHP-1 during HGF stimulation, and the prevention was considered to have been responsible for the lack of mitogenic response to HGF in astrocytes.

Furthermore, astrocytes showed enhanced mitogenic response even 5 h after stimulation with 10% serum (Fig. 2A), which is much shorter than that generally observed in many other cell types. The same profile of mitogenic response was also observed in astrocytes carrying the mutant SHP-1 by the stimulation of HGF (Fig. 8). The rapid mitogenic response of astrocytes may be explained by the fact that primary astrocytes continue to proliferate slowly even in the serum-deprived condition. Thus, it is possible that molecules, except for PLCγ1, involved in progression of cell cycle may be constitutively active in the serum-starved astrocytes and activation of PLCγ1 may lead the cells to immediate response to the mitogenic stimuli.

In contrast to PLCγ1, PI3-kinase was activated by HGF, which in turn induced cell chemokinesis. In the process, activation of PLCγ1 is not essential, since the cells treated by U73122 still showed chemokinetic response to HGF (Fig. 2D). SHP-1 was also associated with PI3-kinase before HGF stimulation, but was dissociated from PI3-kinase immediately after HGF stimulation (Fig. 6B), suggesting that the dissociation of SHP-1 is responsible for the selective activation of PI3-kinase. The activation of PI3-kinase may also contribute to cell proliferation as well as chemokinesis, since wortmannin treatment also inhibited HGF-promoted mitogenic response emerged by mutant SHP-1 (Fig. 8A). These results indicated for the first time that SHP-1 selectively regulates PI3-kinase and PLCγ1 and may determine the astrocyte-specific response to HGF. The rapid dissociation of SHP-1 from PI3-kinase after HGF stimulation contrasts with general observations showing that their association is promoted after stimulation with various cytokines or growth factors (28, 29).

SHP-2, the structurally related molecule of SHP-1 (17), has been reported to be associated with PI3-kinase (30–32), and the expression of SHP-2 predominated over that of SHP-1 in astrocytes (data not shown). However, SHP-2 may not be involved in the selective inhibition of PLCγ1, because that SHP-2 was not associated with PLCγ1 or PI3-kinase in astrocytes (Fig. 6, E and F), and that Cys → Ser mutant of SHP-2 did not induce tyrosine phosphorylation of PLCγ1 (Fig. 7A).

Our results suggest that the selective activation (or suppression) of universal signal mediators, such as PI3-kinase and PLCγ1, is responsible for cell type-specific responses to HGF. As another example of the selective activation of the signal mediators, we have previously reported that HGF induces tyrosine phosphorylation of PLCγ1 and not PI3-kinase in rat primary neocortical neurons (16). Since PI3-kinase was not tyrosine-phosphorylated even in the cells treated with sodium orthovanadate (data not shown), the tyrosine phosphorylation of PI3-kinase was prevented in a manner independent of tyrosine phosphatase. With regard to the differential activation of PI3-kinase by c-Met in COS-7 cells, it has been reported that serine phosphorylation in the juxtamembrane domain of c-Met abolished the binding of PI3-kinase to the receptors and that spliced variant of c-Met lacking this domain facilitated the recruitment of PI3-kinase to c-Met (35, 36). However, only the full-length form was detected in astrocytes and neurons (data not shown). Multiple mechanisms may contribute to the selective activation of PLCγ1 and PI3-kinase according to the cell types.

Our observations revealed the biochemical bases for the selective activation of PI3-kinase and the constitutive suppression of PLCγ1 in astrocytes. However, multiple, cell type-specific mechanisms accounting for the selective activation of diverse subsets of signal mediators may function to exert the pleiotropic effects of HGF. Further studies in this regard may
shed light on the understanding of diverse biological responses of cells to HGF.

Acknowledgment—We are grateful to Dr. Seisuke Hattori, Department of Biochemistry and Cellular Biology, National Institute of Neuroscience, for valuable discussion and comments.

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