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Hepatocyte Growth Factor-Induced Proliferation of Primary Hepatocytes Is Mediated by Activation of Phosphatidylinositol 3-Kinase

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Primary hepatocytes respond to the proliferating signals of Hepatocyte Growth Factor (HGF) through activation of the tyrosine kinase activity of the *met* (p^{145}) receptor. Addition of dHGF in hepatocyte cultures resulted in receptor phosphorylation which co-precipitated with a phosphorylated protein of 85 kDa. This protein was identified as the regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase). Co-precipitation of the PI 3-kinase regulatory subunit with the *met* receptor was observed only with the phosphorylated receptor. Wortmanin, which specifically inhibits PI 3-kinase, was found to abolish the hepatocyte DNA synthetic response due to stimulation with dHGF. It is suggested that the D-3-phosphorylated inositol phospholipids participate as major regulators in the growth and differentiation factor-initiated cascades, this not being restricted to primary hepatocytes.

The recently discovered Hepatocyte Growth Factor (HGF) and its deleted form bearing a deletion of five aminoacids (dHGF) are mitogenic in primary hepatocytes as well as in other cell types such as melanocytes and keratinocytes (1–4). Both HGF and dHGF share the same receptor which was found to be identical with the product of the *c-met* oncogene possessing tyrosine kinase activity (5). Recombinant HGF/SF receptor binds and phosphorylates in vitro, transducers containing src homology region 2 (SH2) domains such as phospholipase-Cγ (PLCγ), rasGAP and a tyrosine kinase of the src family (6). Tyrosine phosphorylation of PLCγ and formation of inositol 1,4,5-trisphosphate ($InsP_3$) by HGF were shown in primary hepatocytes (7). However HGF did not activate PLC γ nor $InsP_3$ production in $HepG_2$ cells where this factor was shown to exert a growth-inhibitory activity (7). Phosphatidylinositol 3-kinase (PI 3-kinase) is a lipid and serine kinase and contains two Src homology 2 (SH2) domains. It is consisted of a regulatory subunit (85 KDa) and a catalytic subunit (110 KDa). Phosphatidylinositol 3-kinase interacts directly with some growth factor receptors resulting in the production of PtdIns (3, 4, 5)P_3 which is a second messenger involved in actin polymerization and in signal amplification (8–10). In our study we describe activation (phosphorylation) of the PI 3-kinase by the met receptor kinase. Activation of PI 3-kinase seemed to be required for the transmission of the HGF proliferating signal in hepatocytes.

MATERIALS AND METHODS

All chemicals were purchased from Sigma Chemical Co. and radioactivity compounds were from Amersham Int., UK. dHGF and anti HGF/dHGF monoclonal antibody was a kind gift of Dr. N. Shima (Tochigi, Japan).

Isolation and culture of primary rat hepatocytes. Hepatocytes from adult rats (male Wistar) were isolated using the two-step collagenase perfusion method (11). The purified hepatocytes were suspended in Dulbecco's Modified Eagle's Medium (DMEM) and plated onto collagen-coated 60mm dishes (Nunc). DMEM was supplemented as described elsewhere (12) and the cells were allowed to attach for two hours and after that period the medium was changed and additions together with growth factors (dHGF) were then made. Hepatocytes were incubated in a humidified 5%CO_2/95% air atmosphere and the medium was thereafter changed every day.

DNA synthesis in hepatocyte cultures. (methyl-^{3}H)thymidine (5μCi/dish, specific activity >85 Ci/mmol), was added to the cultures for two hours. DNA and radioactivity were estimated as previously described (12).

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Western blotting analysis of hepatocyte proteins. Total hepatocyte protein lysates were prepared by resuspending the cells (from 2–3 dishes) in lysis buffer consisting of 20 mM HEPES, 5mM KCl, 5mM $MgCl_2$, 0.5% Triton X-100, 0.1% sodium deoxycholate, 1mM Phenylmethylsulphonyl fluoride, 100μM sodium orthovanadate and 30 μl/ml leupeptin (Sigma). Hepatocyte proteins (25μg/lane) were separated onto a 10% SDS-PAGE and then transferred onto Immobilon-P membrane using a BioRad protein-transfer apparatus. Membranes were blocked with PBS/0.1% Tween 20 containing 5% Bovine Serum Albumin for 30 minutes, washed three times with PBS-Tween 20 at 0.1% and then incubated with the PY-20 anti-phosphotyrosine, anti-PI 3-kinase (Transduction Laboratories) or anti-met antibodies (Santa Crouz). Anti mouse or rabbit-peroxidase-labeled secondary antibody (Dianova) was then added and reactive proteins were revealed using a commercially available ECL kit (Amersham Int.). Stripping of the blots was carried out for 30 minutes at 50°C by incubating the Immobilon filters in Tris-HCl 62.5 mM pH:6.7, 2% SDS and 100 mM β-mercaptoethanol. After stripping the blots were blocked in PBS/Tween-20 0.1%, BSA 5% and ECL procceeded as previously described. Immunoprecipitations were carried out in lysis buffer for 2 hours at 4°C and the immunoprecipitates were collected onto Protein A-agarose (1hr at 4°C) and then eluted after boiling in Laemmli buffer. The immunoprecipitates were subsequently analyzed onto SDS-PAGE as described above.

Assay for InsP3 formation. Hepatocytes were cultured in the presence or not of dHGF and genistein and the levels of InsP3 were assayed as previously described (7) using a commercially available kit (Amersham Int.).

RESULTS AND DISCUSSION

The association of HGF with its receptor (c-met) leads to activation of PLCγ in hepatocytes thus resulting in the production of $InsP_3$ and diacylglycerol serving as second messengers (7), dHGF also induced the production of $InsP_3$ at levels comparable to those observed using HGF (Fig. 1). Pretreatment of the hepatocytes with genistein (a tyrosine phosphorylation inhibitor) inhibited the production of $InsP_3$ which was also abolished by the presence of a monoclonal antibody against HGF/dHGF in the culture (Fig. 1:3–4). Because PI 3-kinase contains SH2 domains thus functioning by binding to tyrosine phosphorylated sequences of the HGF receptor we examined whether this kinase is activated upon stimulation with dHGF. Indeed, in dHGF-treated hepatocyte lysates prepared in lysis buffer containing orthovanadate to prevent receptor dephosphorylation, we identified induction in the phosphorylation of a protein with apparent molecular weight of 85KDa (Fig. 2). Phosphorylation of the 85KDa protein was evident only in dHGF-treated hepatocyte cultures and not in the unstimulated. We then attempted to investigate if the phosphorylated form of the 85KDa protein was associated with the met (p^{145}) receptor. From dHGF stimulated or unstimulated cultures the met (p^{145}) receptor was immunoprecipitated and analyzed onto SDS-PAGE (Fig. 3A). The transferred proteins were then probed with an anti-met antibody and subsequently the blot was stripped and re-probed with a monoclonal antibody against PI 3-kinase. The phosphorylated form of the met receptor ($p^{145\beta}$) was found to co-precipitate with the 85 KDa protein. This protein was identified as the regulatory subunit of PI 3-kinase, indeed reacting with a anti-PI 3-kinase specific monoclonal antibody (Fig. 3B). Pretreatment of the hepatocyte cultures with genistein also abol-

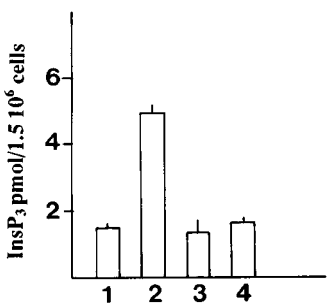

FIG. 1. Effects of dHGF in $InsP_3$ production by primary hepatocytes. Primary hepatocytes were stimulated with dHGF (50ng/ml) for 30 sec. (2). Genistein at 100 μM was added 2 hours before dHGF stimulation (3) and hepatocytes were preincubated with anti-dHGF/HGF monoclonal antibody for 1 hour prior to the addition of dHGF (4). Control levels of $InsP_3$ are shown in (1). Values represent means ± S.E.M. from triplicates from at least four independent experiments.

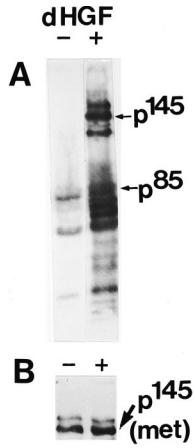

FIG. 2. dHGF stimulates phosphorylation of met receptor in primary hepatocytes. Hepatocytes were stimulated with dHGF (100 ng/ml) for 1 hour. Hepatocyte lysates prepared as described in methods were analyzed onto 10% SDS-PAGE and transferred onto Immobilon-P membranes. The filters were then probed with an anti-phosphotyrosine monoclonal antibody (PY-20) (A) followed by ECL. The filters were stripped and re-probed with an anti-met (B) polyclonal antibody followed by ECL.

ished the appearance of PI 3-kinase in phosphorylated form in lysates from dHGF-treated hepatocytes (data not shown).

Wortmanin is a fungal metabolite and inhibits specifically the IP 3-kinase (13). In hepatocyte cultures treated with dHGF, we examined the effects of wortmanin in the DNA synthetic response induced by the growth factor (Fig.4A). Treatment of dHGF-induced hepatocytes with varying concentrations of wortmanin, resulted in drammatic inhibition of ^{3}H-Tdr incorporation into hepatocyte DNA during all time points. Moreover, wortmanin was effective in inhibiting hepatocyte proliferation at concentrations between 0.1–10μM (Fig. 4B) and its addition to the cultures did not interfere with the viability of the cells, as assessed using the trypan blue exclusion test (not shown).

Inhibition of the PI 3-kinase by wortmanin in insulin-stimulated 3T3-L1 fibroblasts prevented the adipocyte differentiation through down regulation of Ras protein and MAP kinase which act downstream the PI 3-kinase (14). It has also been observed that morphogenesis and motogenesis in inner medullary collecting cells stimulated by HGF, was also inhibited by wortmanin (15). In A549 epithelial cell line and in CTL16 gastric carcinoma cells overexpressing the met receptor, it was shown that the tyrosine phosphorylated receptor (met ($p^{145\beta}$) coprecipitated with the 85 KDa PI 3-kinase subunit (16). The regulatory subunit was associated with the immobilized met receptor

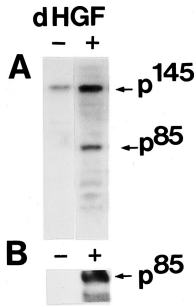

FIG. 3. Phosphorylated met receptor co-precipitates with the regulatory subunit of PI 3-kinase. Hepatocyte lysates from dHGF-stimulated or unstimulated cultures (100ng/ml, 1hr) were immunoprecipitated with an anti-met polyclonal antibody (A), analyzed onto 10% SDS-PAGE and transferred as in Fig. 2. The membranes after stripping were re-probed with anti PI 3-kinase monoclonal antibody (B) and reacting species were revealed by ECL.

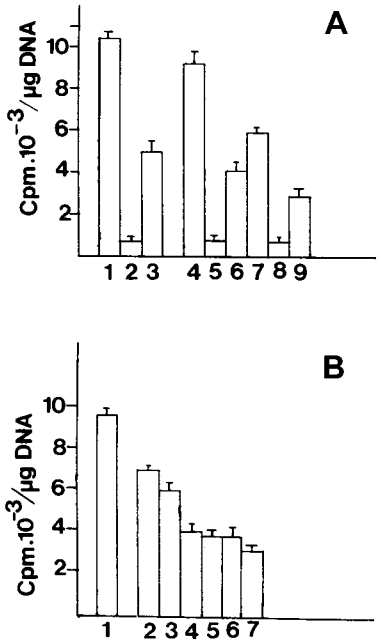

FIG. 4. Effects of wortmanin on dHGF-stimulated hepatocyte DNA synthesis. Wortmanin was added at 2 μM in hepatocyte cultures stimulated with 50 ng/ml of dHGF and 3H-TdR incorporation was followed at 48, 72 and 96 hours postaddition of the factors. (A) (1–3) 48 hrs, (4–6) 72hrs and (7–9) 96 hrs. Bars (2, 5, 8) represent control values from cultures non-stimulated with dHGF. (1, 4, 7) represent values from dHGF-stimulated cultures and (3, 6, 9) represent values from dHGF+ wortmanin-stimulated (4μM) hepatocytes. (B) Wortmanin was added at different concentrations into hepatocyte cultures in the presence of dHGF (50 ng/ml) and 3H-TdR incorporation was detected at 72 hrs. (1) Control incorporation from dHGF-stimulated culture. (2–7) dHGF+ wortmanin at 0.1, 1,2,4,8 and 10 μM, respectively. Values are means from triplicates ± S.E.M. from at least three independent experiments.

which was tyrosine phosphorylated in vitro (17). We have provided evidence that in primary hepatocytes the tyrosine phosphorylated met receptor co-precipitates with the regulatory subunit of PI 3-kinase and this phenomenon was associated with the receptor's phosphorylation state. In addition, inhibition of PI 3-kinase activity by wortmanin resulted in the inhibition of hepatocyte proliferation stimulated by dHGF. It has been suggested that the regulatory subunit of the PI 3-kinase is tyrosine phosphorylated only when associated with cellular membranes, therefore been a target for interactions and in vivo activation by growth factor receptors (18). The IRS-1 (insulin receptor substrate 1) is a target for phosphorylation by the insulin receptor β subunit and was found to be highly phosphorylated on tyrosine residues during the early stages of liver regeneration and also to co-precipitate with the regulatory subunit of IP 3-kinase (19). Growth hormone was also shown to stimulate phosphorylation of the IRS-1 and its subsequent association with the PI 3-kinase in adipocytes (20). These taken together, put the basis for a) a role of the generated D-3-phosphorylated inositol phospholipids in the regulation of hepatocyte proliferation induced by HGF and b) an involvement in the transduction of signals generated by a variety of ligands interacting with receptors.

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