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ROLE OF THE ADENYLATE CYCLASE, PHOSPHOINOSITIDASE C AND RECEPTOR TYROSYL KINASE SYSTEMS IN THE CONTROL OF HEPATOCYTE PROLIFERATION BY HEPATOCYTE GROWTH FACTOR

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Abstract—Hepatocyte growth factor (HGF) is the most potent known mitogen for hepatocytes in primary culture. However, the mechanisms through which HGF induces hepatocyte proliferation have not been defined. Here we have investigated the role of the adenylate cyclase, phosphoinositidase C and tyrosine kinase signalling systems in the control of hepatocyte proliferation by HGF using freshly isolated or cultured adult rat hepatocytes. We show that human recombinant HGF caused a dose-dependent increase in hepatocyte DNA synthesis with a maximal effect at 10 ng/mL and an EC_{50} of 5.9 ng/mL. HGF had no effect on hepatocyte adenylate cyclase activity or intracellular cAMP levels. Elevation of hepatocyte cAMP levels resulted in inhibition of HGF-stimulated DNA synthesis. HGF stimulated inositol phospholipid hydrolysis with a maximal effect at 25 ng/mL and potentiated the effect of vasopressin (10^{-8} and 10^{-9} M). HGF (100 ng/mL) caused an increase in the phosphorylation on tyrosine of an unknown hepatocyte protein with a molecular mass of 36 kDa. Thus, we have shown that HGF, like epidermal growth factor (EGF), can activate the phosphoinositidase C and tyrosine kinase systems in rat hepatocytes. As with EGF, these intracellular signalling systems may underlie HGF-induced hepatocyte proliferation.

Controlled liver cell growth and regeneration allows the restoration of normal liver architecture after injury. Some of the factors that control liver regeneration have now been defined (see Ref. 1 for a recent review). One such factor, hepatocyte growth factor (HGF¶), was first purified from human plasma and rat platelets and was shown to stimulate proliferation of rat hepatocytes *in vitro* 24–48 hr after addition to the culture medium [2–4]. It is a heparin-binding heterodimeric polypeptide that has extensive homology with plasminogen, but no homology with other known growth factors [5]. HGF is the most potent known mitogen for hepatocytes in primary culture [6] and may act as a humoral factor to stimulate liver regeneration *in vivo* (see Ref. 7 for a recent review). Thus, HGF increases in the serum of rats following partial hepatectomy [8], hepatotoxin administration [9] and in patients during fulminant hepatic failure [2, 10]. HGF mRNA increases in rat liver non-parenchymal cells [11, 12] and in lung and kidney in the presence of liver injury, suggesting that HGF may act as a paracrine and an endocrine regulator of hepatocyte growth [7].

Although HGF is a potent stimulus for hepatocyte growth and may mediate liver regeneration following liver injury, the physiological role of HGF is unknown. HGF has now been shown to stimulate proliferation of endothelial cells, and a variety of epithelial cell lines, as well as cultured human melanoma cells [13]. Furthermore, HGF is identical to a scatter factor that stimulates epithelial cell motility [14] and also appears to be similar to a tumor cytotoxic factor produced by a fibroblast cell line [15].

It has now been shown that HGF can have anti-proliferative activity in various tumour cell lines, including a hepatocellular carcinoma cell line [16]. Thus, HGF probably has a diverse role in the control of cell proliferation and tissue repair.

The mechanisms through which HGF induces hepatocyte growth have not been defined. The HGF receptor has recently been identified as the c-*met* proto-oncogene, which is a member of the growth factor receptor tyrosyl-kinase enzyme family [17]. However, HGF-induced activation of tyrosine phosphorylation in liver cells has not been demonstrated and hepatocytes express relatively low levels of HGF receptor [18]. Tyrosine kinase activation by epidermal growth factor (EGF) can result in hepatocyte proliferation [19]. In addition, roles have been proposed for the adenylate cyclase [20] and phosphoinositidase C (PIC) [21]

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¶ Abbreviations: HGF, hepatocyte growth factor; PIC, phosphoinositidase C; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; AVP, arginine vasopressin; IBMX, 3-isobutyl-1-methyl-xanthine; PLC, phospholipase C.

systems in the control of hepatocyte proliferation.

In the present study we have examined the roles of the adenylate cyclase, PIC and tyrosyl kinase systems in the stimulation of hepatocyte DNA synthesis by HGF.

MATERIALS AND METHODS

Materials. Collagenase (Worthington, type II) was obtained from Lorne Laboratories (Reading, U.K.); Williams medium E and fetal calf serum were from Gibco/BRL (Paisley, U.K.); glutamine and penicillin/streptomycin solution were from Flow Laboratories (Irvine, U.K.); [*methyl*-^{3}H]thymidine (44 Ci/mmol) and *myo*-[2-^{3}H]inositol (18.2 Ci/mmol) were from Amersham (Aylesbury, U.K.); mouse EGF, vasopressin, angiotensin II, glucagon and cAMP analogues were from the Sigma Chemical Co. (Poole, U.K.); forskolin was from Calbiochem (Nottingham, U.K.).

Human recombinant HGF was prepared as described previously [5] and was kindly supplied by Dr P. Godowski, Genentech, CA, U.S.A.

Reagents for SDS–PAGE were from Bio-Rad (Hemel Hempstead, U.K.). All other chemicals were of the purest grade available and were either from Sigma or from BDH (Poole, U.K.).

Primary cutlure of hepatocytes. Hepatocytes were isolated from 200–240 g male PVG rats (Bantin and Kingman, Hull) following *in situ* liver perfusion with collagenase [22]. The isolated cells were cultured as monolayers at 37° in 5% CO_2 and 95% air on type 1 collagen-coated 24-well plastic dishes (Falcon) at a density of 6×10^4 cells/well for DNA synthesis and [^{3}H]inositol phosphate experiments. Cells were plated at a density of 5×10^5 on plastic 6-well dishes (Costar) for tyrosine phosphorylation studies. The culture medium used was Williams medium E supplemented with 5% fetal calf serum, 1 nM dexamethasone, 1 nM insulin (tissue culture grade), penicillin (50 U/mL), streptomycin (50 μg/mL) and glutamine (2 mM).

Determination of DNA synthesis. After 20 hr in culture, the medium was changed to serum-free Williams medium E. Growth factors, hormones and cAMP analogues were then added either alone or in combination by a further 20–24 hr. DNA synthesis was determined by labelling cultured cells with [^{3}H]-thymidine (2.5 μCi/well) for a further 6 hr in the presence and absence of 10 mM hydroxyurea according to a previously described method [23].

Assay of adenylate cyclase activity and intracellular cAMP levels. Adenylate cyclase activity was assayed by the method of Salomon [24] using washed plasma membranes prepared from rat hepatocytes as described previously [25]. cAMP was extracted from hepatocytes using perchloric acid and assayed in a competitive binding assay using the regulatory subunit of cAMP-dependent protein kinase [26].

Assay of phosphoinositide metabolism. Hepatocytes were pre-labelled with *myo*-[2-^{3}H]inositol (2.5 μCi/well) for 40 hr in Williams medium E containing 5% dialysed fetal calf serum. Following agonist stimulation, [^{3}H]inositol phosphates were extracted and analysed by a modification of a previously described method [27].

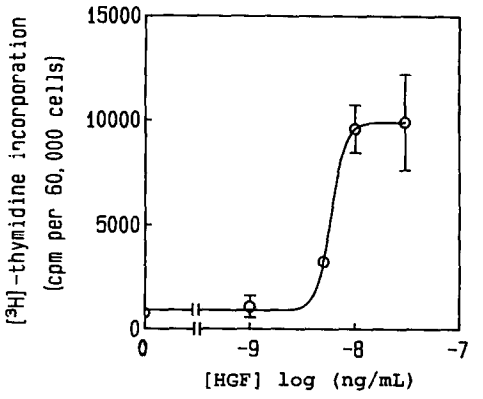

Fig. 1. Effect of HGF on DNA synthesis in adult rat hepatocytes. Hepatocytes were prepared and DNA synthesis was measured as incorporation of [^{3}H]thymidine into cells as described in Materials and Methods. The results are means ± SE from a single experiment performed in triplicate. Similar results were obtained in two different experiments.

Detection of tyrosine phosphorylation. After 20 hr in culture, the medium was changed to serum-free for 5 hr and the cells were then exposed to growth factors for 10 min at 37°. Lysates were prepared by scraping the cells into hot (90°) SDS–PAGE sample buffer [28] containing phosphatase inhibitors [29], followed by snap freezing in liquid N_2.

Hepatocyte proteins were resolved by 8% SDS–PAGE [28] and immunoblotted using a mouse monoclonal antiphosphotyrosine antibody (ICN/Flow, High Wycombe, U.K.) [30].

RESULTS

DNA synthesis

Treatment of primary hepatocyte cultures with HGF caused a dose-dependent increase in DNA synthesis. This effect was maximal with 10 ng/mL HGF and displayed an EC_{50} of 5.9 ± 0.6 ng/mL (mean ± SEM, N = 3, Fig. 1). HGF induced an approximately 7–10-fold maximal increase in DNA synthesis. However, when cells were maintained in culture for more than 48 hr, there was a reduction in responsiveness and the maximum response to HGF was decreased to approximately 3–4-fold although the EC_{50} was unchanged (data not shown).

The maximum increase induced by HGF was similar to that seen in response to EGF (10 ng/mL) (Table 1). Treatment with arginine vasopressin (AVP; 10^{-7} M) or angiotensin II (10^{-7} M), in the absence of other growth factors, had no significant effect on hepatocyte DNA synthesis (Table 1).

Treatment of hepatocytes with agents that elevate cAMP levels [glucagon 10^{-7} M; or 10^{-4} M forskolin plus 10^{-4} M 3-isobutyl-1-methyl-xanthine (IBMX)] or with cAMP analogues (3×10^{-4} M 8-bromo-cAMP; or 3×10^{-4} M dibutyryl cAMP) resulted in

Table 1. DNA synthesis by isolated rat hepatocytes in response to HGF, EGF, AVP and angiotensin II

Treatment	[^{3}H]Thymidine incorporation into DNA (cpm × 10^{-3} per 1.2 × 10^5 cells)	Fold stimulation
Control	7.6 ± 3.1	
HGF (10 ng/mL)	66.5 ± 13.6	8.8
EGF (10 ng/mL)	52.6 ± 19.7	6.9
AVP (10^{-7} M)	11.7 ± 5.3	1.5
Angiotensin II (10^{-7} M)	7.4 ± 1.6	1.0

Hepatocytes were prepared and DNA synthesis was measured as incorporation of [^{3}H]thymidine into cells as described in Materials and Methods.

Results are the means ± SEM of triplicate samples from a single experiment performed twice.

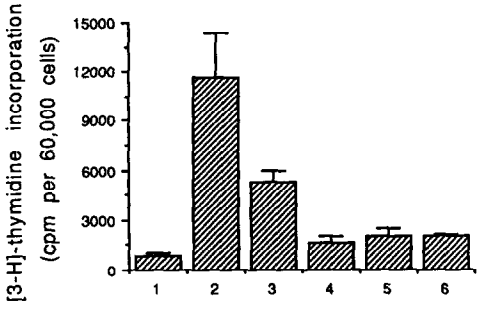

Fig. 2. Effect of elevation of cAMP on HGF-stimulated DNA synthesis in rat hepatocytes. Cells were treated with vehicle (column 1); 10 ng/mL HGF (column 2); 10 ng/mL HGF + 10^{-7} M glucagon (column 3); 10 ng/mL HGF + 10^{-4} M IBMX + 10^{-4} M forskolin (column 4); 10 ng/mL HGF + 3 × 10^{-4} M dibutyryl cAMP (column 5); 10 ng/mL HGF + 3 × 10^{-4} M 8-bromo-cAMP (column 6) and DNA synthesis was measured as described in Materials and Methods. Data represent the means of triplicate samples and SEM from a representative experiment performed twice.

inhibition of HGF (10 ng/mL)-elevated DNA synthesis (Fig. 2).

Adenylate cyclase activity and cAMP levels

In the presence of 100 μM GTP, HGF (10 ng/mL) had no effect on basal adenylate cyclase activity in hepatocyte plasma membranes. The concentration-dependent stimulation of adenylate cyclase by forskolin or glucagon in the presence of GTP were unaffected by HGF (Fig. 3). Similarly, HGF had no effect on the intracellular level of cAMP in cultured hepatocytes and did not affect the increases seen in response to glucagon, forskolin or IBMX treatment (Table 2). The concentrations of glucagon required to activate adenylate cyclase (EC_{50} 22 nM) were very similar to the concentrations of glucagon that inhibited DNA synthesis (IC_{50} 20 nM), not shown.

Inositol phosphate levels

The increase in [^{3}H]inositol phosphates observed in response to HGF was variable. Primary hepatocyte cultures that responded well to AVP (10^{-7} M), i.e. with a greater than 20-fold increase in [^{3}H]inositol phosphate levels, also showed a dose-dependent increase in inositol phosphates in response to HGF, with a maximal effect at 25 ng/mL of approximately 10-fold stimulation (Fig. 4). In hepatocyte preparations that responded poorly to AVP (less than 6-fold increase in [^{3}H]inositol phosphates by 10^{-7} M AVP) HGF had no direct effect on [^{3}H]inositol phosphate levels but was able to potentiate the response to 10^{-8} and 10^{-9} M AVP (Fig. 5).

Tyrosine phosphorylation

Immunoblotting of the primary rat hepatocyte lysate revealed several phosphotyrosyl proteins. HGF (100 ng/mL), or insulin (10^{-7} M) plus EGF (100 ng/mL) caused an increase in tyrosine phosphorylation of a 36 kDa protein. In addition, phosphotyrosyl proteins of approximate molecular masses of 160 and 53 kDa were detected following treatment with insulin plus EGF (Fig. 6).

DISCUSSION

In this study we have shown that human recombinant HGF stimulated DNA synthesis in adult rat hepatocytes in primary culture.

HGF (10 ng/mL = 10^{-12} M) was approximately 10 times more potent than EGF (10 ng/mL = 10^{-11} M) but the maximal DNA synthetic response to HGF and to EGF was the same. When HGF is added in combination with EGF, the maximal synthetic response to these two growth factors is additive [6]. *In vitro*, the rate of liver cell growth is dependent upon the interactions of several different stimulatory and inhibitory factors [1].

When hepatocytes were maintained in culture for an extra day prior to HGF stimulation, there was a significant reduction in DNA synthesis. This could be due to a generalized loss of responsiveness or to a reduction in the number of HGF receptors expressed on the cultured hepatocyte plasma membranes, as occurs for other peptide hormones such as vasopressin [31]. Indeed, many hepatocyte functions are lost during primary hepatocyte culture over a period of 2–4 days [32].

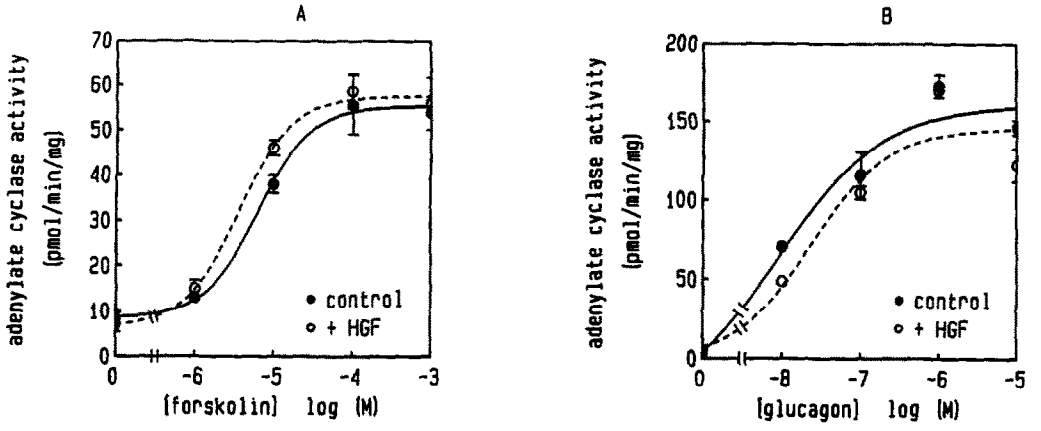

Fig. 3. Effect of 10 ng/mL HGF on the stimulation of hepatocyte adenylate cyclase by (A) forskolin or (B) glucagon. Hepatocyte membranes were prepared and incubated with the indicated concentrations of (A) forskolin or (B) glucagon in the presence (O) or absence (●) of 10 ng/mL HGF. Adenylate cyclase activities were measured as described in Materials and Methods. Data are the means ± range from two separate experiments, each performed in triplicate.

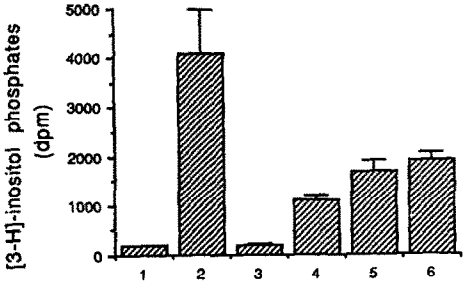

Fig. 4. Effect of AVP and HGF on [^{3}H]inositol phosphates in adult rat hepatocytes. Cells were treated with vehicle (column 1); 10^{-7} M AVP (column 2); 1 ng/mL HGF (column 3); 10 ng/mL HGF (column 4); 25 ng/mL HGF (column 5); 50 ng/mL HGF (column 6) and [^{3}H]inositol phosphates were measured as described in Materials and Methods. Data are the means and SD from a representative experiment performed in triplicate and repeated with similar results.

Table 2. Effect of glucagon, forskolin and IBMX on hepatocyte intracellular cAMP levels

	pmol cAMP per 10^6 cells	
	Control	HGF (10 ng/mL)
Basal	2.1 ± 0.4	3.5 ± 1.5
Glucagon (10^{-7} M)	30.0 ± 0.2	31.7 ± 1.8
Forskolin (10^{-5} M)	10.0 ± 0.9	10.2 ± 0.5
IBMX (10^{-4} M)	4.1 ± 0.1	3.7 ± 0.1

Hepatocytes were prepared, incubated with the indicated concentrations of glucagon, forskolin, IBMX or vehicle in the presence or absence of HGF (10 ng/mL) and cAMP levels were determined as described in Materials and Methods.

Results shown are the means ± SEM from a single experiment performed in triplicate and repeated once with similar results.

We also investigated the cell signalling systems involved in the control of HGF responsiveness using two approaches. First, we examined the effects of HGF on (a) adenylate cyclase activity and cAMP factor-stimulated migration of cultured epithelial and endothelial cells [35, 36].

The HGF receptor has recently been identified as a heterodimeric, transmembrane protein encoded levels; (b) inositol phospholipid hydrolysis and (c) hepatocyte tyrosine phosphorylation. Secondly, we examined the effect of activating these signalling pathways on hepatocyte DNA synthesis in the absence of HGF. Glucagon, forskolin, the phosphodiesterase inhibitor IBMX or cAMP analogues were used to raise the level of cAMP; vasopressin to

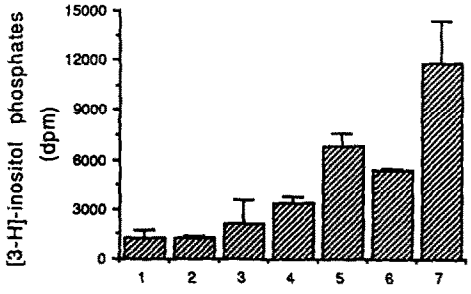

Fig. 5. Effect of HGF and AVP on [^{3}H]inositol phosphates in adult rat hepatocytes. Cells were treated with vehicle (column 1); 10 ng/mL HGF (column 2); 10^{-9} M AVP (column 3); 10^{-8} M AVP (column 4); 10^{-7} M AVP (column 5); 10^{-9} M AVP + 10 ng/mL HGF (column 6); 10^{-8} M AVP + 10 ng/mL HGF (column 7), and [^{3}H]inositol phosphate levels were measured as described in Materials and Methods. HGF significantly enhanced the stimulation of [^{3}H]inositol production by 1 nM ($P = 0.02$) and 10 nM ($P = 0.005$) AVP (data compared using unpaired two-tailed t-test). Data are means and SD from a representative experiment performed in triplicate and repeated with similar results.

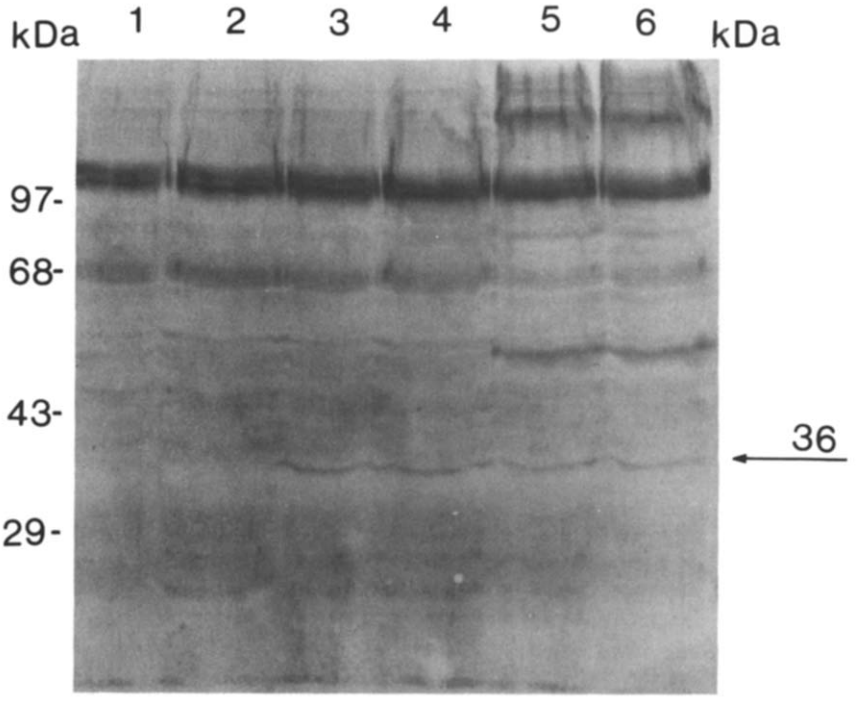

Fig. 6. Western blot showing effect of HGF, EGF and insulin on tyrosine phosphorylation in adult rat hepatocytes. Cells were treated with vehicle (lanes 1 and 2); 100 ng/mL HGF (lanes 3 and 4); 100 ng/mL EGF + 10^{-7} M insulin (lanes 5 and 6) and tyrosine phosphorylation measured as described in Materials and Methods. Results shown are from a representative experiment performed twice.

stimulate the phosphoinositide pathway; and EGF to activate its receptor tyrosine kinase.

The role of cAMP in the control of hepatocyte proliferation is controversial. In primary hepatocyte cultures it has been suggested that cAMP can either stimulate [20] or inhibit DNA synthesis, depending on the concentration used [33]. Furthermore, EGF-stimulated replication of adult rat hepatocytes in primary culture is inhibited by cAMP analogues and agents that increase intracellular cAMP [34].

Our findings indicate that cAMP is not involved in the stimulation of hepatocyte DNA synthesis by HGF since HGF had no effect on adenylate cyclase activity or intracellular cAMP levels in the presence or absence of the phosphodiesterase inhibitor IBMX. Furthermore, elevation of hepatocyte cAMP levels, using various agents, resulted in inhibition of both basal and HGF-stimulated DNA synthesis, indicating a negative regulatory role for cAMP. This is in keeping with studies showing that activators of the adenylate cyclase signalling pathway inhibit scatter by the c-*met* proto-oncogene [17]. Receptor activation by HGF has been shown to cause a rapid increase in tyrosine phosphorylation of the 145 kDa subunit in certain target cells, which express high levels of c-*met* [17, 37]. In the present study, in primary hepatocyte cultures HGF caused an increase in tyrosine phosphorylation of a protein of 36 kDa. Although its identity is, as yet unknown, this protein may be important for mitogenesis in hepatocytes. A phosphotyrosyl protein of 145 kDa, corresponding to autophosphorylated c-*met*, was not identified, probably due to the small number of HGF receptors present on hepatocytes [18]. It is possible that c-*met* is transiently phosphorylated in hepatocytes and that dephosphorylation has occurred during the 10 min incubation period chosen for our studies. Indeed, agonist-induced activation of the hepatocyte insulin receptor tyrosine kinase is rapid and transient under some experimental conditions [38].

The second messengers 1,2-diacyglycerol and inositol 1,4,5-trisphosphate have been shown to be important early mitogenic signals in a number of different cell types and to increase in response to many different mitogens [21, 39, 40]. We have shown that HGF can stimulate inositol phospholipid hydrolysis directly in hepatocytes and potentiate vasopressin-induced increases. In this study, AVP produced a larger increase in phosphoinositide hydrolysis than HGF, but had no direct effect on hepatocyte DNA synthesis. Hormones such as AVP and angiotensin II act as co-mitogens in hepatocytes [1], unlike HGF and EGF which are complete hepatocyte mitogens. Therefore, activation of the PIC pathway is not sufficient *per se* to induce hepatocyte mitogenesis.

Several different isoforms of phospholipase C (PLC) have been purified and cloned [41]. Stimulation of inositol phospholipid hydrolysis by EGF and platelet-derived growth factor (PDGF) has been shown to be the result of phosphorylation on tyrosine of PLC-γ and direct association of the autophosphorylated receptor with the phosphorylated phospholipase [41, 42]. A consensus sequence (-YXXM-) has been proposed for the interaction of receptor tyrosyl kinases with SH-2

domains of proteins such as PLC-γ [43]. This consensus sequence (-Y^{1313}EVM-) is present in the cytosolic domain of the c-*met* protein [44]. It is likely, therefore, that the HGF-induced inositol phosphate production reported here is mediated by the activation of PLC-γ. In a recent study using A549 lung carcinoma cells, Graziani *et al.* [44] demonstrated the direct association of c-*met* with phosphatidyl inositol 3-kinase. Osada *et al.* [45] recently demonstrated activation of phosphatidylcholine–PLC by HGF in rat hepatocytes. Therefore, there is a growing body of evidence to indicate an important role for phospholipid-derived second messengers in the stimulation of cell growth by HGF.

In conclusion, we have shown that HGF-induced mitogenesis is inhibited by cAMP and that HGF can activate both the PIC and tyrosyl kinase systems in rat hepatocytes. These two signalling systems play an important role in the control of hepatocyte growth by HGF.

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