Endocrine, *vol. 9, no. 2, 171–183, October 1998* 0969–711X/98/9:171–183/$11.25

Growth Effects of Regulatory Peptides and Intracellular Signaling Routes in Human Pancreatic Cancer Cell Lines

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The intracellular events involved in normal pancreatic growth have been extensively investigated in response to cholecystokinin. Recent data indicate that tyrosine kinase, phospholipase D, phosphatidylinositol 3-kinase, and p42/p44 MAPK are stimulated in rat pancreatic acinar cells. Although we begin to understand the intracellular signaling pathways activated in normal pancreas, such information is not yet available in pancreatic cancer cells. This study was undertaken to identify the growth factors and hormones involved in cell proliferation of two human pancreatic cancer cell lines of ductal origin, the MIA PaCa-2, and PANC-1 cells, and to establish the intracellular events involved in the control of their growth. We demonstrated that FGF-2, IGF-1, cerulein, and gastrin but not FGF-1, HGF, secretin, and PACAP, stimulated proliferation of MIA PaCa-2 and PANC-1 cells. Autocrine factors such as gastrin and IGF-1 were also responsible for their proliferation. In response to EGF, FGF-2, IGF-1, cerulein, gastrin and bombesin, tyrosine kinase, and tyrosine phosphatase activities were stimulated in both cell lines. The close relationship established between cell growth and tyrosine kinase activation results from the observation that maximal growth stimulation paralleled with maximal enzyme activation and that genistein, the tyrosine kinase inhibitor, blocked cell growth and enzyme activation. The implication of PLD in growth-stimulated processes is doubtful since all growth factors and hormones tested failed to stimulate an already very active PLD activity. We finally observed a constitutive activity of p44 MAPK in both cell lines and of p42 in MIA PaCa-2 cells. However, p38 and p42 were stimulated in MIA PaCa-2 and PANC-1 cells, respectively, by all growth factors and hormones.

Key Words: MIA PaCa-2; PANC-1; EGF; FGF-2; IGF-1; cerulein; gastrin; bombesin; tyrosine kinase; tyrosine phosphatase; PLD; MAPK.

Introduction

The trophic effects of some gastrointestinal peptides on normal pancreatic growth are now well established. Bombesin *(1)*, secretin, and gastrin *(2)*, cholecystokinin (CCK) *(3)* and its structural analog cerulein *(4)* are all mitogenic for the rat pancreas. Other factors like epidermal growth factor can also promote growth of the pancreas *(5)*.

The action of peptide hormones and growth factors on cell growth requires specific interaction with cell surface receptors and subsequent activation of intracellular signaling pathways. The intracellular events involved in pancreatic growth have been extensively investigated recently and especially those in response to CCK, one of the most important regulator of the exocrine pancreas functions. Recent data indicate that occupation of the CCK receptors stimulates tyrosine kinases, phospholipase D (PLD) and phosphatidylinositol 3-kinase (PI 3-kinase) activities in rat pancreas *(6)* and in rat pancreatic acinar cells *(7)*. It was suggested that PLD and PI 3-kinase might be specifically related to the trophic effects of CCK since their activities were not stimulated by carbachol, an acetylcholine agonist with no trophic effect *(8)*. CCK also rapidly activates p42 and p44 MAP kinases *(9)*, known to play pivotal roles in cell proliferation *(10)*, as well as Ras and MEK, the upstream components of the MAP Kinase signaling cascade *(11)*.

Although we are beginning to understand the intracellular signaling pathways activated in the normal pancreas, such information is not yet available for pancreatic cancer cells. Previous studies were designed to understand regulation of human pancreatic cancer cell growth, and to characterize the hormones and growth factors involved in their

Received April 22, 1998; Revised June 15, 1998; Accepted July 13, 1998.
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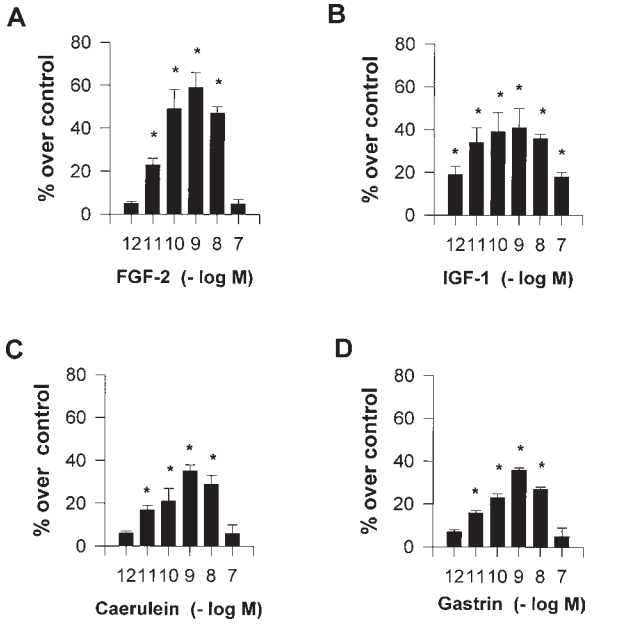
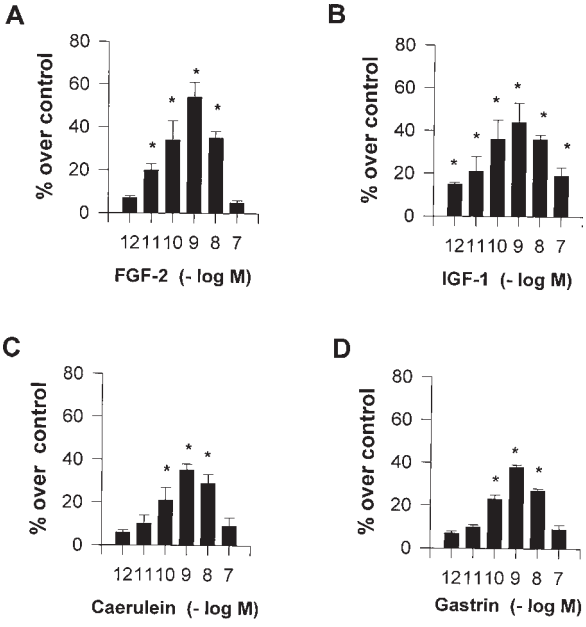


Fig. 1. FGF-2, IGF-1, cerulein, and gastrin-stimulated growth of the MIA PaCa-2 cells. MIA PaCa-2 cells were made quiescent by serum deprivation for 24 h followed by stimulation with increasing concentrations of growth factors and hormones. Agonists were added daily. After 2 d, cells were trypsinized and counted with an Electronic Coulter Counter or a hemacytometer. Values are the mean ± SE of three separate experiments performed in triplicate. *Significantly different from control at $p < 0.05$.



EGF FGF-2

Fig. 2. FGF-2, IGF-1, cerulein, and gastrin-stimulated growth of the PANC-1 cells. PANC-1 cells were made quiescent by serum deprivation for 24 h followed by stimulation with increasing concentrations of growth factors and hormones. Agonists were added daily. After 2 d, cells were trypsinized and counted with an Electronic Coulter Counter or a hemacytometer. Values are the mean ± SE of three separate experiments performed in triplicate. *Significantly different from control at $p < 0.05$.

proliferation. Lierh et al. *(12)* observed that EGF and IGF-1, but not CCK or bombesin stimulated growth of the two human pancreatic cancer cells, MIA PaCa-2 and PANC-1. Growth of the human pancreatic cancer cells MIA PaCa-2, BxPC-3, and PANC-1 was also reported in response to CCK *(13,14)*, although this is contrary to previous data on MIA PaCa-2 and PANC-1 cells *(12)*. Transfection of the cholecystokinin receptors (CCK_A and CCK_B) in MIA PaCa-2 and PANC-1 cells led to their growth inhibition *(15)*.

This study was therefore undertaken to substantiate our information on the growth factors and gastrointestinal (GI) hormones potentially involved in the growth control of two human cancerous pancreatic cell lines of ductal origin, the MIA PaCa-2 and PANC-1 cells, and to establish intracellular signaling pathways specifically involved in the control of their proliferation.

Results

To investigate regulation of human MIA PaCa-2 and PANC-1 pancreatic cancer cells growth, we first established their need for specific growth factors and hormones. Cells were made quiescent by serum deprivation and their proliferation was evaluated in response to increasing concentrations (10^{-12}–10^{-7} *M*) of various hormones and growth factors. As shown in Figs. 1 and 2, FGF-2, IGF-1, cerulein, and gastrin stimulated proliferation of both MIA PaCa-2 and PANC-1 cells. The growth factors were more efficient in both cell lines, whereas cerulein and gastrin were more potent in the MIA PaCa-2 cells (Fig. 1) than in the PANC-1 cells (Fig. 2) with a threshold concentration of 10^{-11} *M* in the MIA PaCa-2 cells. Increasing concentrations of all agonists caused a bell-shaped response in cell numbers with maximal responses obtained at 10^{-9} *M*. Percent increases in cell counts above control values were as follows: in MIA-PaCa-2 cells (Fig. 1), fibroblast growth factor (FGF-2) (+59%), IGF-1 (+41%), cerulein (+35%), and gastrin (+36%); in PANC-1 cells (Fig. 2), FGF-2 (+54%), IGF-1 (+44%), cerulein (+35%), and gastrin (+38%). In a previous study, we demonstrated that EGF and bombesin were also mitogenic for both cell lines *(16)*. It is important to note that FGF-1, hepatocyte growth factor (HGF), secretin, and pituitary adenylate cyclase activating peptide (PACAP) did not influence proliferation of either cell line (data not shown). Even though HGF needs serum for its activation and effects on some cells in culture *(17)*, serum supplement of 0.1 and 1% did not render HGF trophic. The absence of growth response to HGF and PACAP on each cell line also corresponds to the lack of expression of their respective receptor mRNA (data not shown). The failure of FGF-1 to induce growth is surprising because of the growth effect of FGF-2 and the presence of the flg receptor mRNA

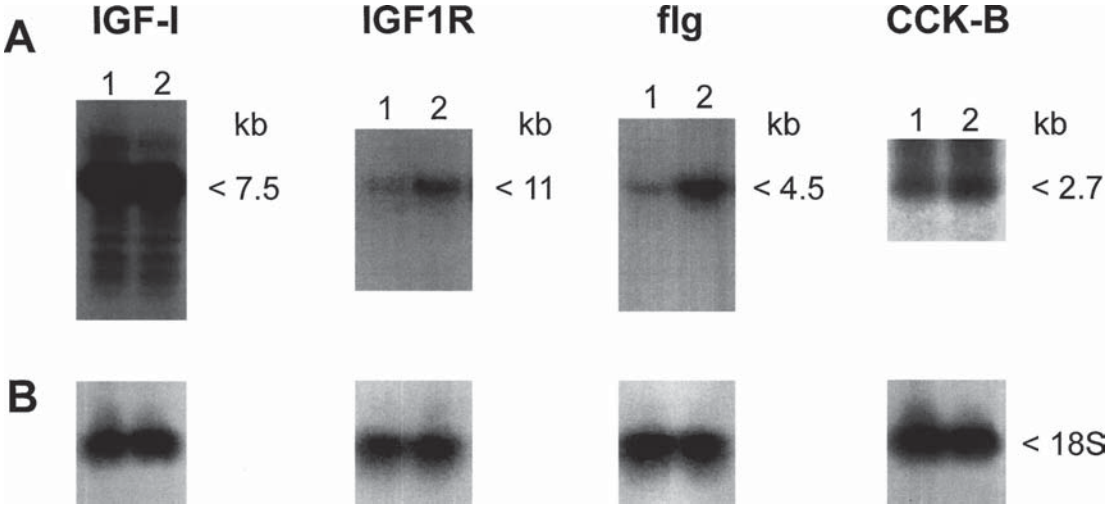


Fig. 3. Growth factors and receptors detected by Northern blots in MIA PaCa-2 and PANC-1 cells. Twenty micrograms of total RNA from both cell lines were blotted onto nylon membranes as described in Materials and Methods. Blots were hybridized using a ^{32}P-labeled cRNA probe **(A)**. Line 1: MIA PaCa-2; line 2: PANC-1. A 18S ribosomal cDNA probe was used to evaluate relative amounts of total RNA transferred to the membrane **(B)**.

Table 1
Effects of IGF-1 and Gastrin Antibodies and CCK_A and CCK_B Receptor Antagonists on Basal, IGF-1, and Gastrin-Stimulated MIAPaCa-2 and PANC-1 Cell Growth[a]

	MIA PaCa-2		PANC-1	
	% of Control	% Difference	% of Control	% Difference
Control	100		100	
Anti-IGF-1 (1/100)	70 ± 5[b]	–30	60 ± 4[b]	–40
Antigastrin (1/100)	70 ± 5[b]	–30	104 ± 3	NIL
Anti-IGF-1 + antigastrin	40 ± 5[b]	–60	63 ± 5[b]	–37
IGF-1 (10^{-9} *M*)	145 ± 3[b]	+45	143 ± 2[b]	+43
Gastrin (10^{-9} *M*)	134 ± 1[b]	+34	135 ± 2[b]	+35
IGF-1 + anti-IGF-1	72 ± 4[c]	–50	63 ± 5[c]	–56
Gastrin + antigastrin	71 ± 6[c]	–47	106 ± 5[c]	–21
IGF-1 + antigastrin	115 ± 5[c]	–21	145 ± 4[b]	NIL
Gastrin + anti-IGF-1	102 ± 4[c]	–24	95 ± 5[b]	–30
Gastrin (10^{-9} *M*)	135 ± 8[b]	+35	136 ± 3[b]	+36
Gastrin + L, 364-718	134 ± 2[b]	+35	137 ± 3[b]	+37
Gastrin + L, 365-260	79 ± 5[c]	–41	100 ± 5[c]	NIL
L, 364-718	102 ± 3	NIL	103 ± 5	NIL
L 365-260	80 ± 3[b]	–20	96 ± 7	NIL

[a]Cells were made quiescent by serum deprivation for 24 h and grown for 48 h with or without 10^{-9} *M* IGF-1 or gastrin, alone or in combination with neutralizing antibodies to IGF-1 or gastrin (1/100 dilution), or with 100 n*M* L, 364-718 or L, 365 260. Stimuli, antibodies, and antagonists were added daily. Values are the mean ± SE of three experiments performed in triplicate.

[b]Significantly different from control at $p < 0.05$.

[c]Significantly different from their corresponding IGF-1 or gastrin-stimulated cells at $p < 0.05$.

(Fig. 3). The unique presence of the CCK_B receptor mRNA subtype on both cell lines (Fig. 3) explains the comparable growth response to cerulein and gastrin. This was further confirmed by the observation that the CCK_B receptor antagonist L-365,260 and not the CCK_A receptor antagonist L-364,718, inhibited gastrin-induced growth (Table 1).

When cells were plated in Petri dishes and transferred to serum-free medium after their attachment, the MIA PaCa-2 and PANC-1 cells grew for at least 2 d under these conditions with the PANC-1 cells growing at a slower pace (data not shown). Such growth under extreme conditions suggests that the cells can produce autocrine factors responsible for their proliferation. Previous studies indicated that

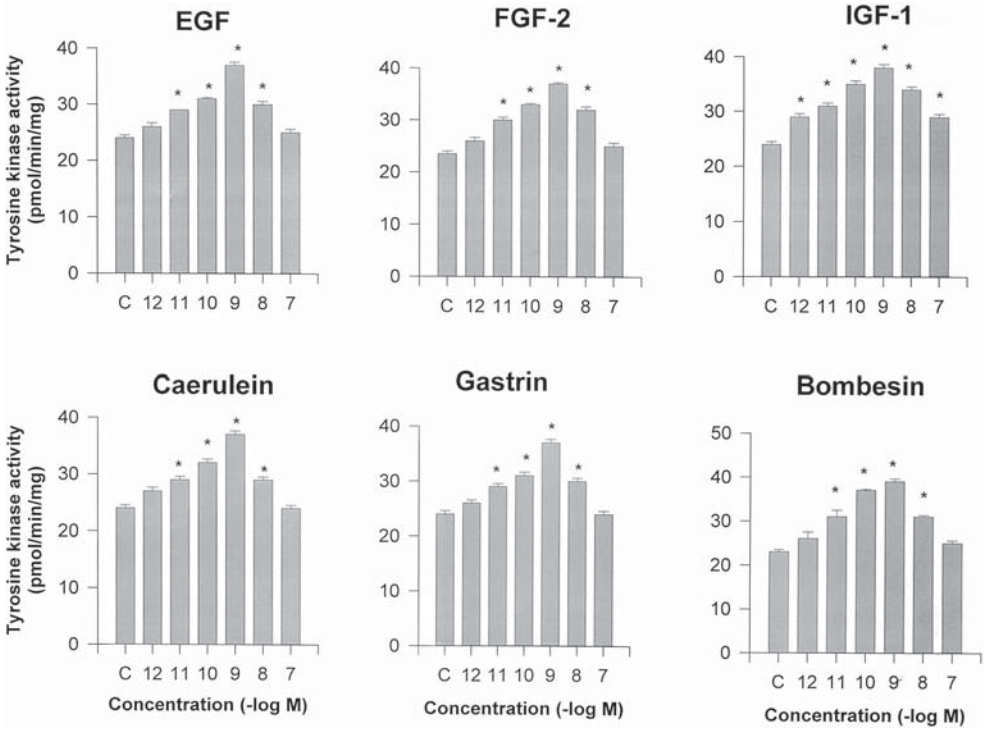


Fig. 4. Tyrosine kinase activity in the MIA PaCa-2 cells. Quiescent cells were incubated with increasing concentrations of EGF, FGF-2, IGF-1, cerulein, gastrin, and bombesin for 30 min. Cells were rinsed with PBS before adding an ice-cold hypotonic lysing buffer. Cells were collected with a rubber policeman and homogenized by repeated strokes. After centrifugation, membranes were resuspended in the lysing buffer and used for tyrosine kinase assays. Tyrosine kinase activity is expressed as pmol of phosphate incorporated/min/mg protein. Values are the mean ± SE of three separate experiments performed in triplicate. *Significantly different from control at $p < 0.05$.

IGF-1 and gastrin can act as autocrine factors for growth of some cancer cells *(18,19)*. In order to establish such an autocrine loop in MIA PaCa-2 and PANC-1 cells, we investigated the effects of IGF-1 and gastrin antibodies and the CCK_B receptor antagonist L-365,260 on basal and IGF-1 and gastrin-stimulated growth. As shown in Table 1, in MIA PaCa-2 and PANC-1 cells, basal growth was significantly reduced by 30 and 40%, respectively, in response to the IGF-1 antibody and by 30% in MIA PaCa-2 cells in response to the gastrin antibody; the PANC-1 cells remained insensible to the gastrin antibody. These data indicate that the MIA PaCa-2 cells can produce IGF-1 and gastrin, whereas the PANC-1 can synthesize only IGF-1. This conclusion is further supported by the observation that combination of the two antibodies further reduced growth of the MIA PaCa-2 cells by 30%, but only the effect of the IGF-1 antibody was observed in the PANC-1 cells, a 37% inhibition comparable to the 40% observed with the IGF-1 antibody alone. Both cell lines responded significantly to IGF-1 (43–45%) and to gastrin (34–35%) for their growth, effect totally neutralized and more by their specific antibody. Indeed, the IGF-1 antibody reduced IGF-1-stimulated proliferation in MIA PaCa-2 and PANC-1 cells by 50 and 56%, respectively, whereas the gastrin-stimulated growth was inhibited significantly by 47 and 21%, respectively, by the gastrin antibody. In PANC-1 cells, the gastrin antibody reduced the effect of exogenous gastrin only down to control level. The importance of endogenous IGF-1 and gastrin in the growth control of both cell lines is further emphasized by the observations that in the MIA PaCa-2 cells, the gastrin antibody significantly reduced the growth-promoting effect of IGF-1 by 21%, and conversely, the IGF-1 antibody reduced the growth effect of gastrin by 24%. In PANC-1 cells, however, the IGF-1 antibody reduced the gastrin effect by 30%, but the gastrin antibody failed to reduce the growth effect of IGF-1, because they do not secrete any gastrin. These data further indicate that the final growth response to IGF-1 or gastrin in MIA PaCa-2 cells grown in serum-free medium have to include participation of their endogenous factor counterpart. This is also true in the PANC-1 cells with exogenous gastrin. The synthesis of IGF-1 in both cell lines was further confirmed by the presence of its mRNA determined by Northern blot (Fig. 3). Finally, the observation that gastrin-stimulated MIA PaCa-2 cell growth was significantly reduced below basal control values by 20% by the CCK_B receptor antagonist indicates the presence of the CCK_B receptor on these cells and strongly supports endogenous release of gastrin. On the contrary, this same antagonist only reduced growth stimulated by exogenous gastrin to basal level supporting the absence of endogenous gastrin release in the PANC-1 cells (Table 1).

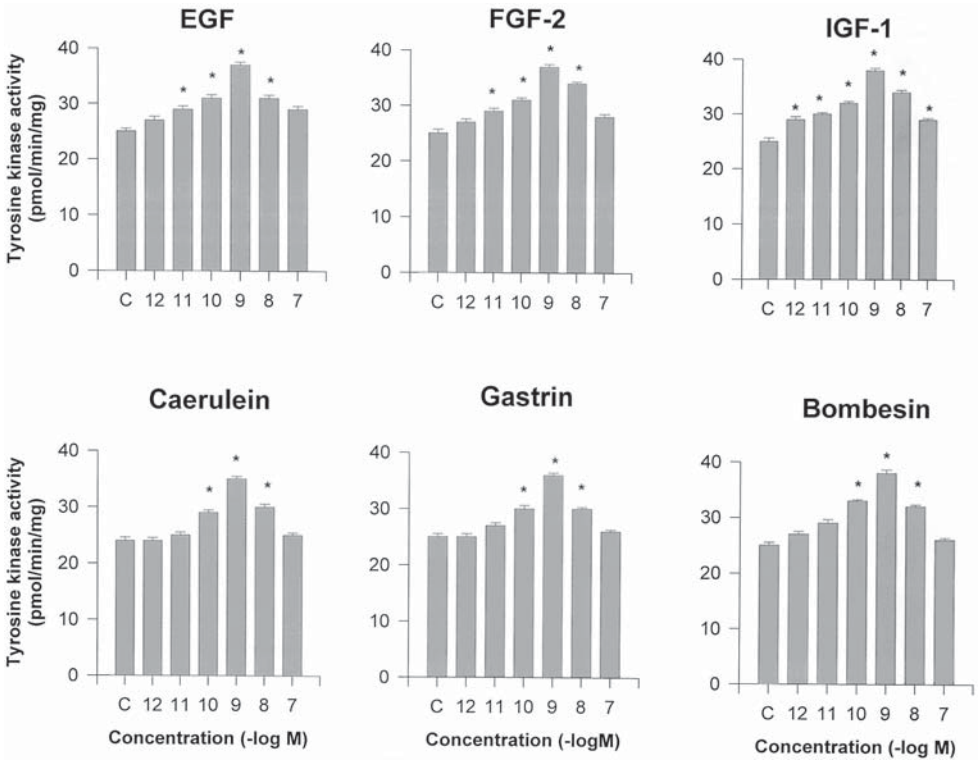


Fig. 5. Tyrosine kinase activity in the PANC-1 cells. Quiescent cells were incubated with increasing concentrations of EGF, FGF-2, IGF-1, cerulein, gastrin, and bombesin for 30 min. Cells were rinsed with PBS before adding an ice-cold hypotonic lysing buffer. Cells were collected with a rubber policeman and homogenized by repeated strokes. After centrifugation, membranes were resuspended in the lysing buffer and used for tyrosine kinase assays. Tyrosine kinase activity is expressed as pmol of phosphate incorporated/min/mg protein. Values are the mean ± SE of three separate experiments performed in triplicate. *Significantly different from control at $p < 0.05$.

Previous observations indicated that tyrosine kinases, tyrosine phosphatases, PLD, and MAPK were associated with growth-related processes *(6,10,20)*. We therefore investigated their potential involvement in MIA PaCa-2 and PANC-1 cell growth by measuring their specific activation in response to EGF, FGF-2, IGF-1, cerulein, gastrin, and bombesin. As shown in Figs. 4 and 5, all stimuli dose-dependently increased tyrosine kinase activities with maximal activation at 10^{-9} *M* in both cell lines and threshold concentrations between 10^{-12} and 10^{-10} *M*. These dose–response curves indicate that the MIA PaCa-2 cells (Fig. 4) are more sensitive to cerulein, gastrin, and bombesin than the PANC-1 cells (Fig. 5) with significant activation of tyrosine kinase at 10^{-11} *M*. Tyrosine kinase activation by all factors in both cell lines was significant within 5 min with a peak activation at 30 min; activation was sustained for 48 h (Table 2). Genistein, a tyrosine kinase inhibitor, also totally inhibited tyrosine kinase activities stimulated by all factors in the MIA PaCa-2 cells along with inhibition of their proliferation (Table 3). A similar response to genistein was observed in the PANC-1 cells with regard to growth and tyrosine kinase activity.

Protein tyrosine phosphatases (PTPase) represent a diverse family of transmembrane receptor-like and non-receptor proteins *(21)*. PTP1C and PTP1D are among the later group and contain Src homology 2 domains (SH2) involved in their association with multiple intracellular signaling molecules, including the growth factor tyrosine kinase receptors *(20,22)*. These two enzymes are often associated with inhibition, PTP 1C *(23)*, or stimulation of cell growth, PTP 1D *(20)*. As shown in Fig. 6, stimulation of the MIA PaCa-2 cells with 10^{-9} *M* of each of the growth factors and hormones, a concentration inducing maximal cell growth, caused significant activation of membrane tyrosine phosphatases (circles). The responses to EGF, FGF-2, and IGF-1 were significant and rapid within 5 min, maximal between 5 and 60 min, but unsustained at 24 h. PTPase activation by cerulein, gastrin, and bombesin was much less important, significant only at 60 min, and is thus transitory. The effects of the growth factors and hormones on membrane tyrosine phosphatase activity were similar in the PANC-1 cells. In all instances, cytosolic tyrosine phosphatase activities (squares) were not affected by treatments in both cell lines.

Recent studies suggested phosphatidic acid, the produce of PLD activation, as a mitogenic signal in some cells *(24,25)*. In the pancreas, PLD is specifically activated by the trophic agent cerulein, but not by the secretagog acetylcholine *(8)*. On its hydrolysis by PLD, phosphatidylcholine gives rise to phosphatidic acid (PA) and free choline. However, PA accumulation can also be derived from

Table 2
Time-Course of Tyrosine Kinase Activation by Growth Factors and Gastrointestinal Hormones[a]

	5 min	30 min	60 min	24 h	48 h
MIA PaCa-2					
Control	24 ± 1	23 ± 1	24 ± 1	24 ± 1	24 ± 0.5
EGF	35 ± 1.4[b]	36 ± 1.3[b]	29 ± 1.4[b]	29 ± 0.5[b]	28 ± 1.2[b]
FGF-2	33 ± 2[b]	36 ± 1.2[b]	29 ± 1[b]	29 ± 0.5[b]	29 ± 0.8[b]
IGF-1	30 ± 1.8[b]	37 ± 1.6[b]	28 ± 1[b]	30 ± 2[b]	29 ± 1.4[b]
Cerulein	28 ± 0.5[b]	34 ± 0.5[b]	29 ± 0.7[b]	28 ± 1[b]	28 ± 3[b]
Gastrin	28 ± 1[b]	38 ± 1[b]	28 ± 0.5[b]	28 ± 1[b]	28 ± 1[b]
Bombesin	35 ± 1[b]	39 ± 1[b]	28 ± 1[b]	29 ± 1[b]	30 ± 2[b]
PANC-1					
Control	25 ± 0.5	24 ± 1	24 ± 0.5	25 ± 0.5	24 ± 0.3
EGF	31 ± 1[b]	34 ± 1[b]	28 ± 0.5[b]	28 ± 0.5[b]	28 ± 1[b]
FGF-2	30 ± 1[b]	35 ± 0.5[b]	27 ± 0.5[b]	29 ± 1[b]	29 ± 1[b]
IGF-1	30 ± 0.5[b]	35 ± 0.5[b]	28 ± 1[b]	29 ± 0.3[b]	29 ± 2[b]
Cerulein	31 ± 1[b]	34 ± 0.6[b]	27 ± 1[b]	29 ± 0.4[b]	29 ± 1[b]
Gastrin	30 ± 1[b]	35 ± 1[b]	27 ± 0.5[b]	29 ± 2[b]	29 ± 1[b]
Bombesin	31 ± 2[b]	38 ± 2[b]	27 ± 0.5[b]	29 ± 1[b]	29 ± 1[b]

[a]Cells were made quiescent by serum deprivation for 24 h and incubated with 10^{-9} *M* of the growth factors or gastrointestinal hormones for the indicated time periods. Cells were then rinsed, lysed, ultracentrifuged, and membranes were collected for the tyrosine kinase assays. Values are the mean ± SE of three experiments performed in triplicate. Results are expressed as pmol of phosphate incorporated/min/mg protein.
[b]Significantly different from control at $p < 0.05$.

Table 3
Effects of Tyrosine Kinase, Tyrosine Phosphatase, and Phosphatidylinositol 3-Kinase and PLD Inhibitors on MIA PaCa2 Cell Growth and Tyrosine Kinase Activity[a]

	Alone		Genistein		Orthovanadate	Wortmannin
	Growth	Tyrosine kinase activity	Growth	Tyrosine kinase activity	Growth	Growth
Control	100	24 ± 1	75 ± 5[b]	18 ± 3[b]	94 ± 4	89 ± 4
EGF	145 ± 3[b]	33 ± 1[b]	79 ± 4[c]	21 ± 3[c]	121 ± 3[c]	120 ± 3[c]
FGF-2	148 ± 7[b]	35 ± 1[b]	81 ± 5[c]	22 ± 2[c]	121 ± 4[c]	121 ± 4[c]
IGF-1	149 ± 5[b]	36 ± 1[b]	85 ± 5[c]	21 ± 1[c]	122 ± 5[c]	125 ± 5[c]
CCK	139 ± 5[b]	33 ± 2[b]	104 ± 4[c]	23 ± 2[c]	125 ± 1[c]	119 ± 6[c]
Gastrin	138 ± 4[b]	35 ± 2[b]	106 ± 7[c]	22 ± 1[c]	126 ± 1[c]	124 ± 7[c]
Bombesin	141 ± 9[b]	36 ± 4[b]	106 ± 6[c]	23 ± 2[c]	125 ± 2[c]	126 ± 5[c]

[a]For the growth study, MIA PaCa-2 cells were made quiescent by serum deprivation for 24 h and incubated for 48 h with 10^{-9} *M* of the growth factors or gastrointestinal hormones, alone or in combination with genistein (1 μ*M*), orthovanadate (1 μ*M*), or wortmannin (50 n*M*). After 2 d, cells were counted with an hemacytometer. Control data represent 100%, and values after treatments represent % of control values. For the tyrosine kinase assays, cells were made quiescent by serum deprivation for 24 h and incubated with 10^{-9} *M* of the growth factors or gastrointestinal hormones for 30 min. Cells were then rinsed, lysed, ultracentrifuged, and membranes were collected for the tyrosine kinase assays. Values are the mean ± SE of three experiments performed in triplicate and represent pmol of phosphate incorporated/min/mg protein.
[b]Significantly different from control at $p < 0.05$.
[c]Significantly different from their respective control stimulus at $p < 0.05$.

diacylglycerol kinase activity *(26)*. The key reaction distinguishing between both activities remains the exclusive transphosphatidylation property of PLD with a primary alcohol *(27)*. As shown in Table 4, growth factors, hormones, Phorbol-12-myistate-13-acetate (PMA), and serum failed to increase PLD activity significantly in both cell lines as measured either by PA accumulation, phosphatidylethanol, or phosphatidylbutanol production. The PLD activity seems maximal under basal conditions and higher in PANC-1 than in MIA PaCa-2 cells. This high basal PLD activity remained insensitive to inhibitors, such as genistein, calphostin-C, lovastatin, and wortmannin. To ascertain that

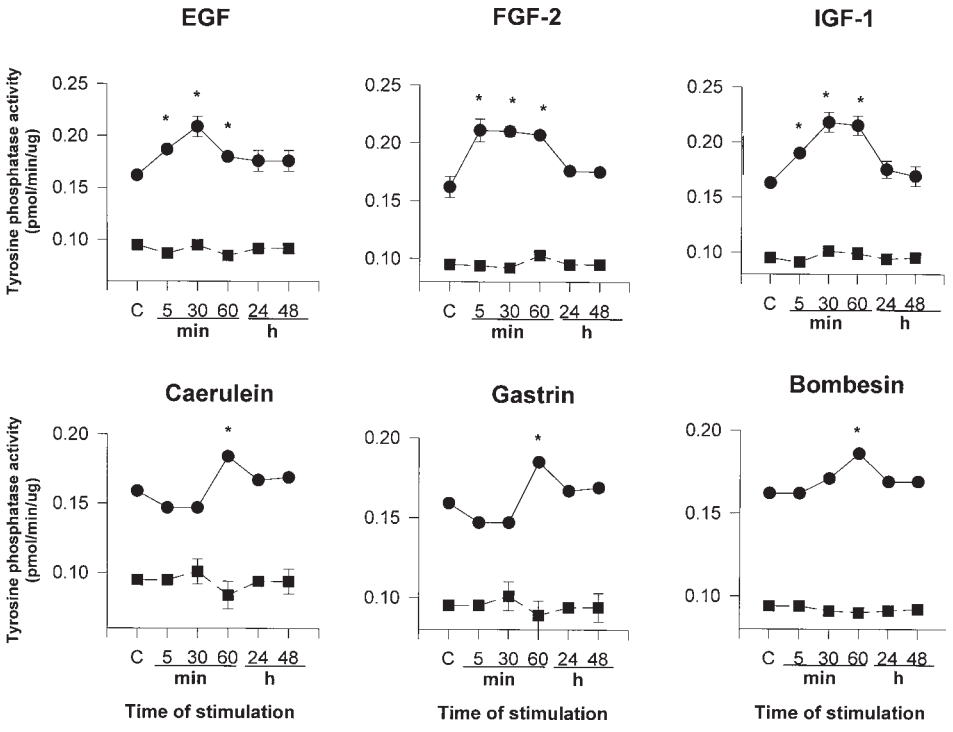


Fig. 6. Tyrosine phosphatase activity in the MIA PaCa-2 cells. Quiescent cells were incubated with increasing concentrations of EGF, FGF-2, IGF-1, cerulein, gastrin, and bombesin for 5 min to 48 h. Cells were rinsed with PBS before adding an ice-cold hypotonic lysing buffer containing no orthovanadate. Cells were collected with a rubber policeman and homogenized by repeated strokes. After centrifugation, membranes were resuspended in the lysing buffer and used for tyrosine phosphatase assays. Tyrosine phosphatase activity is expressed as pmol of phosphate release/min/μg protein. Values are the mean ± SE of three separate experiments performed in triplicate. *Significantly different from control at $p < 0.05$. Circles represent membrane tyrosine phosphatase activity, whereas squares represent cytosolic tyrosine phosphatase activity.

Table 4
Effects of Growth Factors and Gastrointestinal Hormones on PLD Activation[a]

	MIA PaCa-2			PANC-1		
	PA %	PEt %	PBut %	PA %	PEt %	PBut %
Control	0.86	0.25	0.20	1.21	0.39	0.31
EGF	0.88	0.24	0.19	1.16	0.38	0.31
FGF-2	0.81	0.23	0.21	1.18	0.40	0.32
IGF-1	0.84	0.23	0.21	1.17	0.38	0.29
Cerulein	0.86	0.26	0.21	1.16	0.39	0.29
Gastrin	0.82	0.25	0.22	1.20	0.38	0.30
Bombesin	0.87	0.24	0.21	1.17	0.37	0.30
Serum	0.88	0.25	0.20	1.17	0.39	0.31
PMA	0.85	0.24	0.19	1.20	0.39	0.30
Calphostin-C	0.87	0.26	0.20	1.19	0.38	0.31
Lovastatin	0.87	0.26	0.21	1.21	0.39	0.32
Genistein	0.88	0.24	0.20	1.21	0.37	0.31
Wortmannin	0.87	0.25	0.21	1.20	0.38	0.30
Acini control	ND	0.13	ND	ND	0.13	ND
Acini cerulein	ND	0.20[b]	ND	ND	0.20[b]	ND

[a]Cells grown in six-well plates were labeled with 2 μCi/mL of ^{3}H-myristic acid for 24 h in serum-free medium. The cells were then incubated with 10^{-9} *M* of the growth factors, gastrointestinal hormones, 100 n*M* PMA, or 10% serum for 15 min, and processed as indicated in Materials and Methods for PLD assay. For the experiments with drugs, cells were incubated for 30 min at 37°C. For the experiment with the pancreatic acinar cells, cells were labeled for 1 h, washed, and then incubated with 5×10^{-10} *M* cerulein for 15 min at 37°C. Values are the mean ± SE of three experiments performed in triplicate. Results are expressed as percentage of total phospholipids.

[b]Significantly different from control at $p < 0.05$. ND, not determined; PA, phosphatidic acid; Pet, phosphatidylethanol; PBut: phosphatidylbutanol.

the absence of PLD activation in pancreatic cancer cells was not owing to technical problems, normal rat pancreatic acinar cells were stimulated by cerulein in the presence of 1% ethanol. As shown in Table 4, cerulein stimulated PLD activity by 54%, indicating that the enzyme can be activated in normal cells. Furthermore, we can appreciate that basal activity in acinar cells is much less than that in both cancer cell lines.

In order to establish that these specific intracellular enzymes are closely associated with growth of the two cell lines, specific inhibitors of their activity were investigated in response to EGF, FGF-2, IGF-1, cerulein, gastrin, and bombesin stimulation. As indicated in Table 3, the tyrosine kinase inhibitor genistein significantly reduced basal cell growth of the MIA PaCa-2 cells by 25%. Genistein was more efficient in inhibiting the proliferative effects of EGF, FGF-2, and IGF-1 (45%) than that of the GI hormones cerulein, gastrin, and bombesin (23%) in these MIA PaCa-2 cells. Inhibition of basal and stimulated tyrosine kinase activity by genistein corresponds to this inhibitor's reduction of basal and stimulated cell growth in the MIA PaCa-2 cells. The tyrosine phosphatase inhibitor orthovanadate and the PI-3-kinase-PLD inhibitor wortmannin were equipotent in inhibiting all growth factors and hormone-stimulated growth by about 14% in the MIA PaCa-2 cells. Similar responses to genistein, orthovanadate, and wortmannin were observed in the PANC-1 cells regarding their growth and tyrosine kinase activity.

In line with activation of membrane tyrosine kinase and tyrosine phosphatase are the cytosolic MAP kinases whose activation has been associated with cell proliferation *(10)*. As shown in Fig. 7A,B, in MIA PaCa-2 cells, p44 and p42 kinases exhibit constitutive activities with very high basal levels and no response to stimulation. On the contrary, the p38 kinase shows a time-course activation up to 60 min in response to FGF-2 and cerulein (Fig. 7B), and a maximal activation within 5 min in response to IGF-1 and gastrin (Fig. 7A). In the PANC-1 cells (Fig. 7C,D), p44 remained constitutively active and unresponsive to stimulation, and p38 presents very low basal activity and no response to stimulation. In these cells, however, p42 presents a biphasic activation with peak activity at 5 and 60 min in response to FGF-2, cerulein, and gastrin (Fig. 7D). The response to IGF-1 was already maximal after 5 min and sustained for 60 min (Fig. 7C).

Discussion

In this study, we observed that the human pancreatic cancer cells MIA PaCa-2 and PANC-1 grew significantly within 2 d in response to the growth factors FGF-2 and IGF-1 and the GI hormones gastrin and the CCK analog cerulein. An autocrine loop involving IGF-1 and gastrin was also demonstrated. We established an existing relationship between stimulated growth responses and activation of specific intracellular signaling pathways. Indeed, all growth factors and GI hormones testing positive for proliferation significantly stimulated at comparable concentrations tyrosine kinase, tyrosine phosphatase, and selective MAP kinases. Furthermore, inhibition of tyrosine kinase activity also resulted in a return to basal growth rates or less; inhibition of tyrosine phosphatase and PI3-kinase also reduced growth factor-stimulated cell growth, but to a lesser extend.

Previous studies indicated that FGF-2 mediated cell growth in AR4-2J, a rat acinar pancreatic cancerous cell line *(28)* and stimulation of DNA synthesis in rat pancreatic acini *(29)*. Our data support such trophic effects of FGF-2, this time on two human pancreatic cancerous ductal cell lines. Although FGF-1 shares 53% sequence homology with FGF-2 *(30)* and is believed to act through the same receptor *(31)*, it failed to induce growth of our two cell lines, even though FGF-1 was shown to be equally efficacious as FGF-2 and 10-fold less potent in stimulating DNA synthesis in rat pancreatic acini *(29)*. The reported effects of CCK and gastrin on pancreatic cancer cells growth are controversial. Proliferation of the MIA PaCa-2 and PANC-1 cells was observed in response to CCK *(13)* but the GI hormone failed to do so in another study *(12)*. More recently, many positive observations came out implicating occupation of the CCK_B receptor subtype in the growth control of these MIA PaCA-2 and PANC-1 cells as shown by Heald et al. *(14)*, Smith et al. *(13)*, and Kaufmann et al. *(32)*. The present study strongly supports a direct action of CCK and gastrin in the growth control of the MIA PaCa-2 and PANC-1 cells via occupation of the CCK_B receptors. These two cell lines grew identically in the presence of CCK and gastrin, failed to proliferate in the presence of the CCK_B receptor antagonist L-365,260, and possess the CCK_B receptor mRNA. Finally, L-365,260 also inhibited basal growth of the MIA PaCa-2 cells, but not that of PANC-1 cells confirming the gastrin autocrine loop in the MIA PaCa-2 cells.

Differences existing among pancreatic cancer cells and normal cells are also emphasized in this study. PACAP induced AR4-2J cells proliferation *(33,34)*, but failed to do so in the MIA PaCa-2 and PANC-1 cells in which the PACAP receptor mRNAs were not detected. HGF is a potent mitogen for the human pancreas *(35)*; its receptor, the product of the c-met protooncogene, is expressed in normal rat pancreas *(36)*, in AR4-2J cells, and in the human pancreatic carcinomas cells Panc Tu-1 and 818/4 *(37)*. In the MIA PaCa-2 and PANC-1 cells, HGF had no proliferative effect, and we could not detect any c-met mRNA.

All growth factors and GI hormones involved in the growth control of the MIA PaCa-2 and PANC-1 cells lighted up some similar intracellular enzymatic signals, tyrosine kinase, tyrosine phosphatase, and selective MAP kinases. The rapid and sustained stimulation of membrane tyrosine kinase activities by growth factors and GI hormones supports their direct implication in the growth pro-

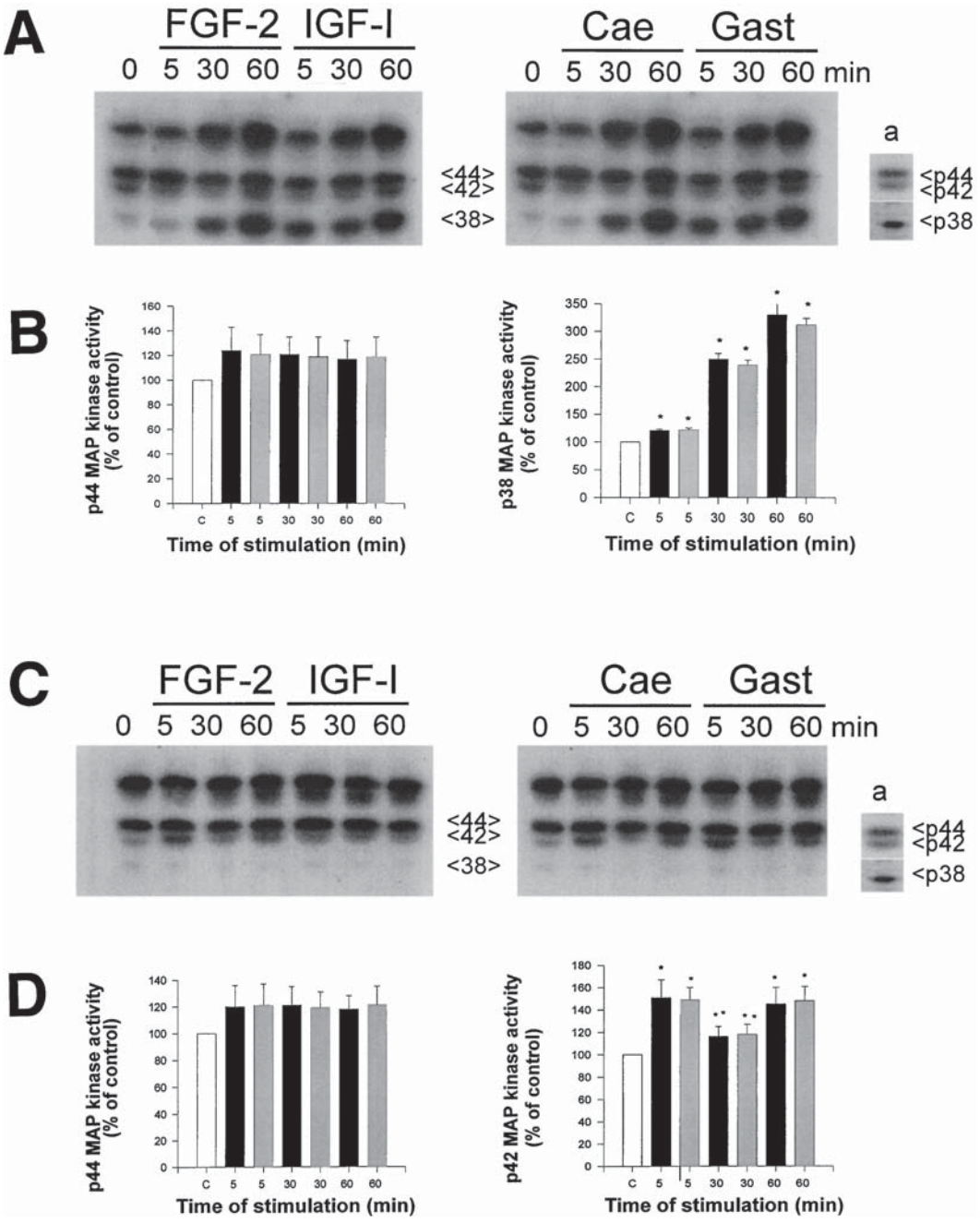


Fig. 7. Identification and activities of MAPKs in MIA PaCa-2 and PANC-1 cells. MIA PaCa-2 **(A)** and PANC-1 **(C)** were made quiescent by serum deprivation. Cells were then incubated with 10^{-9} *M* FGF-2, IGF-1, cerulein, and gastrin for 5, 30, and 60 min. Time zero represents the basal activity. A and C present a representative "in gel" MAP kinase assay. Cells were lysed and subjected to electrophoresis for in gel assay (lanes 0–60) or for Western blotting with anti-p44, p42, and p38 antibodies (lane a) (**B**: MIA PaCa-2) and (**D**: PANC-1): Quantification of p44, p42, or p38 activities in response to 10^{-9} *M* FGF-2 (black bar) and 10^{-9} *M* cerulein (gray bar). Results are expressed as percentage of control values (white bar) and represents the mean ± SE of three separate experiments *Significantly different from control at $p < 0.05$. **Significantly different from FGF-2 and cerulein-stimulated MAPK at $p < 0.05$.

cesses. Such rapid and sustained activation of tyrosine kinases was also observed in rat pancreas in vivo in response to cerulein *(6)*. The close relationship established between cell growth and tyrosine kinases activation in this study results from the observation that maximal stimulation of both events occurred at the same stimuli concentrations and that genistein, a tyrosine kinase inhibitor, comparatively blocked cell growth and kinases activation by all stimuli.

In this study, overall PTPase activity was increased, but not sustained in response to all stimuli, and maximal activity was reached 30 min earlier with the growth factors than with the GI hormones. Such activation was totally blocked by orthovanadate, a specific inhibitor, which also partially

inhibited the growth response to all stimuli, suggesting PTPase implication in the control of cell growth. Some nonreceptor PTPases are most likely involved, and those include PTP1C and PTP1D. A major implication of PTP1C in the MIA PaCa-2 and PANC-1 cells is doubtful, because PTPase activity was stimulated by all stimuli in the MIA PaCa-2 cells in which PTP1C is not expressed (data not shown). In both cell lines, we cannot yet ascertain a participation of PTP1D as activator of specific intracellular enzymatic pathways, but we have detected its presence in both cell lines without being able to estimate any of its activity. According to Rivard et al. *(38)*, the enzyme has to be overexpressed to detect its activity. If PTP1D is involved in any growth processes, it could operate as a binding site for the Grb2-Sos complex *(39)*, *ras* activation down to the MAP kinases as observed in 293 cells stimulated by EGF *(40)*.

The role of PLD in the overall control of cell growth is not yet fully clarified. In rat pancreas, PLD has been associated with the trophic effect of CCK *(6)*, and was activated by EGF, FGF-2, and IGF-1 *(41)*. In MIA PaCa-2 and PANC-1 cells, all growth factors and GI hormones tested failed to stimulate an already active PLD under basal conditions. This relatively high basal PLD activity, resistant to hormonal stimulation and to inhibition by genistein and wortmannin (Table 4), suggests that we may have a constitutive PLD activity like the p44 MAP kinase previously described in these two cell lines. Further studies are needed to clarify this very active and unresponsive PLD in both cell lines. PKC-activated PLD was investigated, since growth factor-induced PLD activation was shown to be PKC-dependent in fibroblasts *(42,43)*. As shown in Table 4, in our two cell lines, PKC does not seem to be involved in PLD activation, since the enzyme did not respond to the phorbol ester PMA and its basal activity was not reduced by the PKC inhibitor calphostin-C. Ras proteins have been implicated in the induction of PLD activation, since *ras* transformation is associated with increased PLD activity in fibroblasts *(44,45)*. In our cell lines, *ras* is transformed by mutation *(46)* and therefore may cause a constitutive activated PLD. Such a possibility is doubtful, since lovastatin, an inhibitor of *ras* farnesylation, responsible for its activation, did not reduced PLD activity (Table 4), although it totally inhibited growth of both cell lines.

MAP kinases p44 (ERK-1) and p42 (ERK-2) activation has been observed in normal rat pancreatic acinar cells in response to CCK *(9)* and in the pancreatic acinar cancer cells AR4-2J in response to CCK and gastrin *(47)*. However, the two kinases exhibit a different and unusual pattern of activation in MIA PaCa-2 and PANC-1 cells in response to all growth-associated stimuli. The constitutive activity of the p44 kinase in both cell lines and of p42 in the MIA PaCa-2 cells is unique and has never been reported before. Of great interest are the findings that these activities cannot be stimulated by any stimulus (Fig. 7), or inhibited by the specific inhibitors used in this study, genistein and wortmannin, or by the specific MEK-1 inhibitor PD98059 *(16)*. The other unusual behavior of the MIA PaCA-2 cells remains that their p38 kinase, a stress kinase, is time-dependently activated by all stimuli. Its implication in cell proliferation has not yet been determined in the MIA PaCa-2 cells, but its activation has been previously associated with T-cell proliferation *(48)*, with T-cell HIV-1 replication *(49)*, and with intestinal wound repair *(50)*. Its activation has also been reported in rat pancreatic acini in response to CCK *(51)*.

The data presented in this study clearly indicate that growth of the MIA PaCa-2 and PANC-1 cells presents a common selectivity for specific growth factors and GI hormones. Furthermore, their respective intracellular signals leading to proliferation seem identical regarding tyrosine kinases and tyrosine phosphatases activation, but differ at the MAP kinases level. In MIA PaCa-2 cells, p38 activation seems involved, whereas p42 would interact in the PANC-1 cells. The role played by PLD remains unclear, and further studies remain to be done for its clarification.

Materials and Methods

Chemicals

The following reagents were purchased. EGF, FGF-2, IGF-1, and HGF were obtained from Collaborative Research, Mississauga, Ontario. Bombesin and gastrin were from Peninsula, Laboratories, Belmont, CA. Cerulein was a gift from R. de Castiglione, Farmitalia, Milan, Italy. Wortmannin was a gift from Sandoz Co, Montreal Qc. Genistein, orthovanadate, Myelin Basic Protein, were from Sigma, St. Louis, MO. Calphostin-C, lovastatin, and PMA were from Calbiochem, San Diego, CA. Penicillin-streptomycin, amphotericin-B, Dulbecco's Modified Eagle Medium (DMEM), and fetal bovine serum were purchased from Gibco-BRL, Burlington, Ontario. ^{32}P-ATP, ^{3}H-myristate, ^{32}P-UTP, and ^{32}P-dCTP were from Amersham, Arlington Heights, IL. Anti-IGF-1 antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Antigastrin was from Incstar Corporation, Stillwater, OK. Tyrosine kinase and tyrosine phosphatase kits were from Boehringer Mannheim, Montreal, Canada. Ready Organic scintillation mixture was from Beckman, Mississauga, Ontario. Phosphatidylethanol and phosphatidylbutanol were from Avanti Polar Lipids Inc., Alabaster, AL. L-364,718 and L-365,260 are gifts from Merck Sharp and Dohme, Westpoint, PA. Anti-p44 and p38 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, and Anti-p42 was a gift from Fergus McKenzie, Université de Nice, Nice, France.

Cell Culture

MIA PaCa-2 and PANC-1 pancreatic carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in DMEM containing 10% fetal bovine serum, penicillin-streptomycin, and glutamine. Cells were cultivated at 37°C in humidified air containing 5% CO_2.

Growth Assays

All experiments were performed starting with confluent cells that were subsequently plated for growth assay in 35-mm diameter dishes (10 cm^2 at 1×10^4 cells/mL, 2 mL/dish. After the attachment phase, cells were transferred to serum-free medium and allowed to become quiescent overnight. The next day, fresh serum-free medium was added, and the cells were then supplemented daily for 2 d with growth factors or hormones at concentrations from 10^{-12} to 10^{-7} *M*. Cell growth was measured after 2 d with an electronic coulter model 2-m counter and with an hemacytometer. Genistein (1 μ*M*), wortmannin (50 n*M*), and orthovanadate (1 μ*M*), which inhibit, respectively, tyrosine kinase, PLD, and tyrosine phosphatase were also used in combination with growth factors and hormones to evaluate the involvement of these enzymes in the growth processes of the MIA PaCa-2 and PANC-1 cells.

RNA Preparation and Northern Blotting

Total RNA was isolated by the procedure of Chomczynski and Sacchi *(52)*.

Preparation of the Probes

Each complementary RNA (cRNA) probe of each factors and receptor was transcribed from the respective cDNA fragment inserted into the plasmid vector pBluescript SK II (Stratagene, La Jolla, CA). The templates were linearized, and T3 or T7 RNA polymerase was used for in vitro antisense transcription. After linearization, cRNA probes were labeled with [^{32}P]UTP for Northern blots using Promega Transcription Riboprobe System (Promega, Madison, WI). The IGF-1 cRNA probe was transcribed from the 800-bp fragment of mouse IGF-1 codant region inserted into the plasmid vector pSP72. The templates were linearized with *Hin*dIII, and T7 RNA polymerase was used for antisense transcription. The IGF-1 receptor cRNA probe was transcribed from the 700-bp fragment of human IGF-1 receptor codant region inserted into the plasmid vector pBluescript KS II+. The templates were linearized with *Sac*II, and T3 RNA polymerase was used for antisense transcription. The FGF receptor cRNA probe was transcribed from the 1113-bp fragment of rat FGF receptor codant region inserted into the plasmid vector pBluescript SK II+. The templates were linearized with *Bam*HI, and T7 RNA polymerase was used for antisense transcription. The CCK_B receptor cRNA probe was transcribed from the 2240-bp fragment of rat CCK_B codant region inserted into the plasmid vector pGEM-3Zf+. The templates were linearized with *Xba*I, and T7 RNA polymerase was used for antisense transcription.

Northern Blot Analysis

Twenty micrograms of total RNA (quantitated by measurement of absorbance at 260 nm) were size-fractionated on a 1% agarose gel containing 2.2 *M* formaldehyde and transferred to nylon membranes (Nytran Plus, Schleicher and Schuell, Keene, NH) as described by Sambrook et al. *(53)*. Hybridization was performed at 65°C for 16–20 h in the above solution containing [^{32}P] UTP RNA probe (1×10^6 cpm/mL). After hybridization, filters were exposed to Kodak X-Omat AR film (Eastman Kodak Company, Rochester, NY) at –80°C for variable periods of time. An 18 S ribosomal [^{32}P]dCTP-labeled cDNA probe was used as a control probe to evaluate RNA loading and transfer.

Preparation of Plasma Membrane Fractions for Tyrosine Kinase and Tyrosine Phosphatase Activities

Cells were grown in Petri dishes (100 mm) until they reached 75% confluency. Cells were then serum-starved for 24 h, and treated for 5, 30, 60 min, and 24 and 48 h with EGF, FGF-2, IGF-1, cerulein, gastrin, and bombesin at a concentration of 10^{-9} *M*. After the indicated time periods, cells were rinsed with PBS before adding an ice-cold hypotonic lysing buffer containing 10 m*M* HEPES, pH 7.2, 5 m*M* KCl, 1 m*M* DTT, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 μ*M* aprotinin, and 2 μ*M* leupeptin with 100 m*M* orthovanadate for the tyrosine kinase assay. Cells were collected with a rubber policeman, homogenized by repeated strokes, and centrifuged at 1000*g* for 5 min at 4°C. Supernatants were collected and ultracentrifuged at 50,000*g* for 30 min at 4°C. Membranes were resuspended in the lysing buffer, and used for protein, tyrosine kinase, and tyrosine phosphatase assays. When inhibitors were used, cells were pretreated for 30 min with each inhibitor and then stimulated for 30 min with each stimulus in the presence of the appropriate inhibitor. At the end, cells were processed as described above.

Tyrosine Kinase and Phosphotyrosine Phosphatase Activities

Tyrosine kinase and tyrosine phosphatase activities were measured using a nonradioactive kit from Boehringer Mannheim (Montreal, Quebec). Briefly, enzymes activities were measured by incubating a synthetic peptide substrate (corresponding to amino acids 6–20 of the cell division kinase p34 cdc2, which is biotin-labeled at the amino-terminus) or a phosphopeptide substrate with ATP/Mg, 5X assay buffer (0.25 *M* Tris-HCl, pH 7.8, with or without 500 μ*M* orthovanadate and 25 m*M* mercaptoethanol) and 10 μg of cell membrane for 30 min at 37°C. The reaction is stopped, an aliquot of the reaction mixture is transferred to a microplate, and the phosphorylated or dephosphorylated substrate is immobilized by binding to the streptavidin-coated microplate. After subsequent washes, the fraction of phosphorylated or dephosphorylated substrate is determined immunochemically with a highly specific antiphosphotyrosine antibody directly conjugated to peroxydase. The absorbance is measured at 405 nm with a reference wavelength at 490 nm using a microplate reader. The results are compared to a standard curve and are expressed as phosphate incorporated (tyrosine kinase) or phosphate release (tyrosine phosphatase).

Phospholipase D Activity

Phospholipase D activity was measured according to the method of Hess et al. (1997). Cells were plated in six-well plates until they reached 75% confluency. The cells were serum-starved in DMEM supplemented with 0.5% fatty-acid-free bovine serum albumin for 24 h before the start of the assay. For the final 16 h of serum starvation, the cells were labeled with 2 μCi/mL ^{3}H myristic acid. At the start of experiment, the cells were washed three times with phosphate buffered saline (PBS) and pre-equilibrated at 37°C in serum-free medium for 1 h. For the final 10 min of preincubation, 0.3% butanol or 1% ethanol was added. At the end of the preincubation, cells were treated with the indicated growth factors and hormones for 15 min. Incubations were terminated by removing the medium, washing once with PBS, and adding 1 mL of ice-cold methanol. Cells were scrapped off the plates, and the lipids extracted and separated with methanol/chloroform/0.1 *N* HCl (1:1:1). The lower phase was dried under N_2, resuspended in 40 μL of chloroform/methanol (95:5) containing phosphatidic acid, phosphatidyl-butanol, or phosphatidylethanol standards, and spotted onto silica gel 60A thin-layer chromatography plates. The plates were developed in the upper phase of the solvent system of ethyl acetate/iso-octane/H_2O/acetic acid (55:25:50:10) and stained with Coomassie blue. Phosphatidic acid, phosphatidylbutanol, or phosphatidylethanol bands were scraped into scintillation vials containing 500 μL of methanol and 10 mL of Ready Organic scintillation mixture. Radioactivity incorporated into specific and total phospholipids was measured.

Preparation of Pancreatic Acini

Pancreases from rats fasted overnight were removed and trimmed of fat and mesentery. A suspension of pancreatic was prepared as reported by Peikin et al. *(54)*.

Preparation of Cytosolic Fractions for MAPK Activity

Cells were grown in petri dishes (100 mm) until they reached 75% confluency. They were then serum-starved for 24 h, and treated for 5, 30, and 60 min with the different stimuli at a concentration of 10^{-9} *M*. When drugs were used, cells were pretreated for 30 min before stimulation with the agonists. After stimulation, cells were rinsed with PBS before adding an ice-cold hypotonic lysing buffer containing 10 m*M* Tris-HCl, pH 8.0, 5 m*M* KCl, 1 m*M* DTT, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, 1 μ*M* aprotinin, 2 μ*M* leupeptin, and 100 μ*M* orthovanadate. Cells were collected with a rubber policeman, homogenized by repeated strokes, and ultracentrifuged at 100,000*g* for 30 min at 4°C in a Beckman TL 100 centrifuge (rotor, TLS55). The cytosolic fractions were collected. After the addition of Laemmli buffer, samples were boiled 5 min before the MAP kinase assays. An aliquot of the supernatant was kept for protein assay determined according to Bradford *(55)*.

MAP Kinases in Gel Assay

MAP kinases activity was determined in renatured sodium dodecyl sulfate (SDS) polyacrylamide gels according to the method of Kameshita and Fujisawa (1989). Briefly, cell extracts (20 μg protein) were resolved on a 10% SDS-polyacrylamide gel copolymerized with 0.25 mg/mL myelin basic protein (MBP). After electrophoresis, gels were washed with four changes of 50 m*M* Tris, pH 8.0, containing 20% propanol. The gels were then denatured with two changes of 60 min each of 120 mL denaturating buffer containing 6 *M* guanidine hydrochloride, 50 m*M* Tris, pH 8.0, and 5 m*M* mercaptoethanol. The enzymes on gel were then renatured with four changes (2 × 60 min, 1 × overnight and 1 × 60 min) of 250 mL renaturating buffer containing 50 m*M* Tris, pH 8.0, 0.4% Tween-20, and 5 m*M* mercaptoethanol at 4°C. The renatured gels were then incubated in an assay buffer containing 40 m*M* HEPES, pH 8.0, 10 m*M* $MgCl_2$, 2 m*M* DTT, and 0.1 m*M* EGTA at room temperature for 30 min. The MAP kinase activities were determined by incubating the gels into 20 mL of the assay buffer containing 20 μ*M* ATP and 100 μCi ^{32}P-ATP at room temperature for 2 h. The reaction was then stopped by adding 250 mL of a solution containing 5% trichloroacetic acid and 10 m*M* sodium pyrophosphate, followed by washing with the same solution nine times over a period of 1.5 h to eliminate nonspecific radioactivity in the gels. Gels were exposed to Kodak X-OMAT film overnight at –70°C before development. Quantification of the MAP kinase activity was carried out with a scanning densitometer (Bio-Rad Imagin Densitometer model GS-670).

Statistical Analysis

Results were analyzed by Student's *t*-test. Results were considered significantly different from control at $P < 0.05$.

Acknowledgments

This research was supported by grants from Natural Science and Engineering Research Council of Canada (GP6369), from the Medical Research Council of Canada (MT 13203), and from le Ministère de l'Éducation du Québec (ER1092). A. Lajas was on a fellowship from Junea De Extremadura and Z. Coulombe had a Canadian Association of Gastroenterology/Hoechst Marrion Roussel Summer Research Initiative Award.

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