Signal Transduction Pathways Involved in the Mitogenic Activity of Pleiotrophin

IMPLICATION OF MITOGEN-ACTIVATED PROTEIN KINASE AND PHOSPHOINOSITIDE 3-KINASE PATHWAYS

Boussad Souttou, Shakeel Ahmad, Anna T. Riegel, and Anton Wellstein

From the Lombardi Cancer Center and Department of Pharmacology, Georgetown University, Washington, D. C. 20007

Pleiotrophin (PTN) is a developmentally regulated protein which exhibits neurite-outgrowth, mitogenic, and angiogenic properties. It has also been shown to be involved in tumor growth and metastasis. Here we used primary BEL (bovine epithelial lens) cells to investigate the signal transduction pathways involved in the mitogenic activity of recombinant PTN. PTN was purified from conditioned media of SW-13 cells transfected with the human PTN cDNA. We show that inhibitors of tyrosine kinase, mitogen-activated protein kinase, or phosphoinositide (PI) 3-kinase inhibit DNA synthesis stimulated by PTN. Analysis of tyrosine-phosphorylated proteins following PTN stimulation showed phosphorylation of two novel 190- and 215-kDa proteins in addition to SHC, ERK1, and ERK2. A mobility shift of phosphorylated ERK1 and ERK2 was detected with a panERK antibody confirming the phosphorylation of the two ERKs. Furthermore, in vitro immunocomplex kinase assay with Akt1, a natural substrate of PI 3-kinase, showed an activation of the kinase following PTN stimulation and a reversal by the PI 3-kinase inhibitor wortmannin. We conclude that the mitogenic activity of PTN is dependent on tyrosine kinase activation and utilizes the mitogen-activated protein kinase and the PI 3-kinase pathways to transduce a mitogenic signal.

MATERIALS AND METHODS

Reagents—Orthovanadate, genistein, tyrphostin A25, and pertussis toxin were purchased from LC Laboratories (Woburn, MA). Wortmannin was from Sigma. PD98059 was obtained from Biolabs (Beverly, MA). [3H]Thymidine and [γ-32P]ATP were purchased from Amersham (Buckinghamshire, United Kingdom). Myelin basic protein was obtained from Boehringer Mannheim (Germany). Bovine basic fibroblast growth factor (bFGF) was obtained from Collaborative Research (Bedford, MA). Anti-PTN antibody was a generous gift of Dr. J. Courty (Universite Paris 12, France) and routinely grown in fibroblast growth factor family of heparin-binding growth factors which display several overlapping biological properties (reviewed in Ref. 6).

The mitogenic activity of PTN is a subject of controversy as conflicting results were reported by different laboratories. On one hand, PTN purified from tissues or expressed in bacteria and in insect cells is devoid of mitogenic activity but still promotes neurite outgrowth (7–9); however, Courty et al. (10) reported mitogenic activity after purification of PTN from bovine brain. On the other hand when expressed in eukaryotic cells, PTN is capable of stimulating thymidine incorporation and cell growth in different cell systems including fibroblasts (11), epithelial cells (11, 12), and endothelial cells (11, 13). The failure of purifying mitogenically active PTN in the former systems listed above may be explained by either loss of mitogenic activity during the purification or erroneous folding of the protein when highly expressed. This latter explanation would be in agreement with the findings of Larrubbi et al. (13) who demonstrated that PTN expressed in NIH3T3 cells is produced in two forms distinct in their structure and properties. These two forms can be separated in two peaks on a Mono S column; the first peak containing mitogenically active PTN and the second peak containing non-mitogenic PTN. Thus the mitogenic activity of PTN would depend on the balance between the active form(s) and the non active form(s). Further support of the role of PTN in cell growth is provided by experiments showing that transfection of the PTN cDNA in SW13 cells and NIH3T3 cells conferred a growth advantage in soft agar and tumorigenesis in nude mice (11, 14). In addition, recent results from our laboratory show that depletion of PTN from human melanoma and choriocarcinoma cells with ribozymes inhibits tumor growth, invasion, angiogenesis, and metastasis (15–17). Taken together these results point to an important role of PTN in cell proliferation and differentiation as well as in tumor progression. In this report we study the signal transduction pathways involved in the mitogenic activity of PTN and show an implication of the MAP kinase and PI 3-kinase pathways.

1 The abbreviations used are: PTN, pleiotrophin; BEL, bovine epithelial lens cells; bFGF, basic fibroblast growth factor; ERK, extracellular signal regulated kinase; MEK, MAP/ERK kinase; PI 3-kinase, phosphoinositide 3-kinase; MAP, mitogen-activated protein; PAGES, polyacrylamide gel electrophoresis; PY, phosphorylated tyrosine.
Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2.2 g/liter sodium bicarbonate (Life Technologies, Gaithersburg, MD), and 1 ng/ml bFGF. PTN overexpressing SW13 cells (SW-13/PTN cells clone W8), produced by stable transfection with the pRcCMV vector containing the PTN cDNA (11), were maintained in IOsev's modified Dulbecco's medium supplemented with 10% FCS (Life Technologies).

**Thymidine Incorporation Assay**—This assay was carried out essentially as described in Ref. 12. BEL cells were seeded in 24-well plates for 2–3 days in Dulbecco's modified Eagle's medium supplemented with 10% FCS and 2.2 g of sodium bicarbonate (Life Technologies). The cells were then serum starved for 24 h after which test samples were added. In the experiments involving inhibitors of signal transduction, the cells were pretreated with the drugs for 1 h prior to adding the growth factors. The cells were then incubated for 18 h at 37 °C and 5% CO2 and then [3H]thymidine was added. After an additional incubation period of 6 h the cells were fixed with 10% trichloroacetic acid, washed with water, and lyzed overnight with 0.3 M NaOH. Total radioactivity incorporated was counted using a Beckman scintillation counter. Each experiment included buffer or vehicle as controls.

**Purification of PTN from Conditioned Media**—Conditioned media from approximately 109 SW-13/PTN cells grown for 4 to 5 days in 1.5 liters of Dulbecco's modified Eagle's medium, 2% FCS was adjusted to 50 mM Tris-HCl, pH 7.5, 0.5 mM NaCl and was passed through a 2-mL heparin-Sepharose column (Pharmacia, Piscataway). The column was then washed with 40 mL of 50 mM Tris-HCl, pH 7.5, 0.5 mM NaCl and NaCl and heparin-bound proteins were eluted with 10 mL of 50 mM Tris-HCl, pH 7.5, 1 mM NaCl. The eluate was diluted to 50 mM Tris-HCl, pH 7.5, 0.25 mM NaCl and passed through a Mono S column using an fast protein liquid chromatography system (Pharmacia). The column was washed extensively in the same buffer containing 0.45 mM NaCl and the bound proteins eluted using a gradient from 0.45 to 2 mM NaCl. Fractions of 1 mL were collected, quickly aliquoted, and stored at −80 °C.

**Western Blot**—After separation in SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Bio-Rad) for 2 h at 150 mV. Western blots were then washed in phosphate-buffered saline, 0.1% Tween 20, 5% powdered milk and probed with antibodies at appropriate dilutions for 1 h at room temperature. The blots were then washed in phosphate-buffered saline, 0.1% Tween 20 and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Amersham) for 1 h. After additional washing in phosphate-buffered saline, 0.1% Tween 20, bound antibody was visualized using the enhanced chemiluminescence reagents system from Amersham.

**Immunoprecipitations**—Cells were grown to 80% confluency in 15-cm dishes, serum starved for 48 h, and then stimulated with PTN or bFGF for 10 min. Cell lysates were prepared by scraping the cells in immunoprecipitation (IP) buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.5% deoxycholic acid, 1% Nonidet P-40, 1% sodium dodecyl sulfate, 1 mM sodium orthovanadate, 1 mM nicotinic acid, 50 mM sodium fluoride, 2 μM leupeptin, 2 μM aprotinin, 1 μM pepstatin A) and incubating for 15 min at 4 °C in a rotating rack. The lysates were cleared by centrifugation and protein content was measured with the Bio-Rad protein assay kit. 0.5 to 1 mg of protein were incubated overnight at 4 °C with 20 μl of 4G10 anti-phosphotyrosine antibody coupled to agrose beads (UBI). The beads were then washed in IP buffer and proteins were eluted by boiling in SDS-PAGE sample buffer and subjected to electrophoresis and Western blotting.

**Immunocomplex Kinase Assay**—Cell lysates, prepared as described above and precleared with protein G-Sepharose, were incubated for 4 h at 4 °C with 3 μg of sheep anti-Akt1 antibody (UBI). The immunocomplexes were captured with protein G-Sepharose at 4 °C for 1 h. The beads were then washed with 50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol and the kinase assay was carried out as described in Ref. 18 with a slight modification: the beads were resuspended in a kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 5 μM ATP, 1 μM protein kinase A inhibitor peptide, 25 μg/ml myelin basic protein, and 2 μCi [γ-32P]ATP) and incubated for 30 min at 30 °C. The reaction was stopped by addition of 5 × sample buffer and boiling. Samples were then electrophoresed, transferred to a nitrocellulose membrane, and the membrane processed for autoradiography.

**Statistical Analysis**—Unless stated otherwise data points were run in triplicate and experiments repeated at least twice. Typically the mean ± S.E. from a representative experiment is presented. As appropriate, Student's t test or ANOVA was used to assess the statistical significance of differences between measurements (Statview 4.02 program; Abacus Concepts Inc.; Berkeley, CA). The respective p values are given in the text. p < 0.05 was considered significant.

**RESULTS**

**Purification and Mitogenic Activity of PTN**—PTN was purified from 1.5 liters of SW-13/PTN conditioned media by a two-step procedure. The first step involved heparin-Sepharose chromatography and elution with a buffer containing 1 mM NaCl. For the second step of purification, the 1 mM NaCl eluate was diluted to 0.25 mM NaCl and applied to a Mono S column on fast protein liquid chromatography. PTN was then eluted from the column with a gradient of NaCl. PTN eluted from this column at 0.75 mM NaCl. The fractions containing the peak were analyzed by SDS-PAGE and silver staining for purity and by Western blot to identify PTN. As shown in Fig. 1A, a >95% purification was achieved. Two major bands of apparent molecular mass of 14 and 18 kDa were detected by silver staining and were identified as PTN by Western blotting (Fig. 1, A and B). Although elution of the two PTN forms appears to overlap, it is obvious from Fig. 1A that the 14-kDa protein is eluted first and thus has a slightly lower affinity for the column. This result is consistent with a truncation of positively charged amino acids. As the C terminus of the protein is rich in lysines, it is likely that the 14-kDa PTN protein represents a C-terminally truncated form of PTN as demonstrated using an anti-PTN antisera raised against a 10-mer peptide from the N terminus of PTN.2

---

2 A. Wellstein, unpublished data.
The mitogenic activity of PTN was assayed on serum-starved and growth-arrested BEL cells which have been shown to be stimulated by this growth factor (12). Fig. 1C shows that the heparin-Sepharose 1 m NaCl fraction and Mono S fractions 2 and 3 induced thymidine incorporation by 5–6-fold. Fraction 1 contained only weakly active material and fraction 4 showed no activity. Conditioned medium from cells transfected with the empty pRc/CMV vector processed for purification under the same conditions showed no mitogenic activity (data not shown). These results indicate that the purified PTN was a mixture of mitogenic and non-mitogenic proteins. Since the mitogenically active form eluted first from the column, it is conceivable that mitogenic activity is mostly due to the truncated protein. The Mono S fraction number 3 was used in the following experiments.

The Mitogenic Activity of PTN Is Independent from the Mitogenic Activity of bFGF—It has been demonstrated previously that bFGF is a potent mitogen for BEL cells (12). To rule out the possibility of contamination of the PTN fractions by bFGF produced endogenously by SW-13 cells (19) and to demonstrate that the mitogenic activity of PTN is independent from that of bFGF, we carried out the experiments shown in Fig. 2. A dose-response curve of bFGF is shown in Fig. 2B. The plateau of stimulation was reached at 1 ng/ml bFGF. When BEL cells were stimulated with bFGF or PTN in the presence of anti-bFGF neutralizing antibodies, only the bFGF activity was reduced (Fig. 2A) demonstrating that PTN is not contaminated by bFGF. Finally, when BEL cells were stimulated with very high concentrations of bFGF (2 and 10 ng/ml), addition of PTN still stimulated the cells above the stimulation achieved by bFGF alone (Fig. 2C). This result indicates that the effects of PTN and bFGF on DNA synthesis are mutually independent and suggests that the PTN receptor is different from the FGF receptor.

Effects of Inhibitors of Tyrosine Kinase, MEK-1, and PI 3-Kinase on the Mitogenic Signal of PTN—To determine which signal transduction pathway(s) is(are) used for mitogenic signaling by PTN, we initially utilized different inhibitors of signal transduction and bFGF, as well as serum, as positive controls. bFGF is known to signal through membrane receptors with tyrosine kinase activity (20) and serum contains a mixture of factors that use different signal transduction pathways.

Pertussis toxin had no effect on DNA synthesis stimulated by PTN and bFGF, but markedly inhibited DNA synthesis induced by serum (Fig. 3A). This result indicates that the mitogenic signals of PTN and bFGF are not mediated via pertussis toxin-sensitive G proteins. The tyrosine kinase inhibitors genistein and tyrphostin A25 strongly inhibited DNA synthesis induced by PTN, bFGF, and serum, whereas the tyrosine phosphatase inhibitor orthovanadate did not (Fig. 3A). Rather, orthovanadate slightly enhanced PTN-, bFGF-, and serum-induced thymidine incorporation. These results indicate that PTN like bFGF stimulates DNA synthesis through tyrosine kinase pathways and that probably the PTN receptor is not a tyrosine phosphatase. Interestingly, tyrphostin A25 was less potent against PTN than against bFGF since 2 μM of this inhibitor inhibited the bFGF effect by more than 60% and the PTN effect by less than 30% (p < 0.01 for inhibition of PTN versus inhibition of bFGF). This suggests that some of the tyrosine kinases involved in the signaling of both growth factors are distinct.

The effects of inhibitors of the MAP kinase and the PI 3-kinase pathways on PTN activity were also studied. The MEK-1 inhibitor PD98059 inhibited PTN-, bFGF-, and serum-induced DNA synthesis in a dose-dependent manner (Fig. 3B). Interestingly, PTN-stimulated mitogenesis was more sensitive than bFGF- and serum-stimulated mitogenesis. The PTN effect was completely inhibited at 10 μM of the inhibitor whereas a less than 50% inhibition was seen for the other two factors. Similarly, the PI 3-kinase inhibitor wortmannin strongly inhibited the PTN effect (by 75%; p < 0.01 for PTN versus control) and less efficiently the bFGF effect (by 35%; p < 0.01 for bFGF versus control; Fig. 3C). Serum-induced DNA synthesis was not affected by wortmannin at 10 nM (Fig. 3C). Taken together, these results indicate that the MAP kinase and the PI 3-kinase pathways are involved in the mitogenic signal of PTN as well as of bFGF. The apparent selectivity of different inhibitors signifies that the coupling of the signaling steps in the PTN and bFGF pathways are distinct and/or that additional pathways may be used by bFGF and not by PTN. Furthermore, the incomplete inhibition of DNA synthesis by wortmannin indicates that mitogenesis is only partly dependent on a functional PI 3-kinase pathway.

PTN Stimulates Protein Tyrosine Phosphorylation—In BEL cells PTN stimulates tyrosine phosphorylation of a predominant 190-kDa protein as assessed by Western blotting of total cell lysate or after immunoprecipitation and Western blotting with an anti-phosphotyrosine antibody (Fig. 4, top). A less prominent 215-kDa protein is tyrosine phosphorylated in addition to the 190-kDa species (Fig. 4, bottom, and Fig. 5, top). The phosphorylation of p190 and p215 was specific for PTN since both proteins were not phosphorylated after bFGF stim-

![Fig. 2. Comparison of PTN and bFGF mitogenic activities.](image-url)
ulation (Fig. 4, bottom) although ERK1 and ERK2 were found tyrosine phosphorylated in response to bFGF (data not shown). In addition, several low molecular weight proteins are tyrosine phosphorylated following PTN stimulation. These proteins have apparent molecular masses of 66, 52, 46, 44, and 42 kDa corresponding to the 3 molecular forms of SHC and to ERK1 and ERK2, respectively (Fig. 5, bottom). A Western blot for SHC (Fig. 5, bottom) and for ERK (see Fig. 6) confirmed that indeed SHC and ERK1/ERK2 were phosphorylated after PTN stimulation of the cells. Pretreatment of the cells with the PI 3-kinase inhibitor wortmannin did not influence phosphorylation of any of the above proteins as expected from the separate signaling pathways.

To independently confirm phosphorylation of ERK1 and ERK2 following PTN stimulation, cell extracts from unstimulated and stimulated cells were analyzed by Western blotting using a panERK antibody. After PTN stimulation a discrete increase in upward-shifted forms of ERK1 and ERK2 were observed in the Western blots (Fig. 6). This mobility shift is typical of phosphorylated forms of the ERKs. Upon closer inspection a single band of ERK1 was detected in the control cells corresponding to the non-phosphorylated form. Following PTN stimulation, all of the ERK1 was shifted to an apparently higher molecular mass suggesting complete phosphorylation of ERK1 (Fig. 6). For ERK2, a doublet was detected in the control cells corresponding to both non-phosphorylated and phosphorylated forms. Upon PTN stimulation, all of the non-phosphorylated ERK2 shifted to the higher mobility form as expected.

**Fig. 3.** Effect of signal transduction inhibitors on the mitogenic activity of PTN. Serum-starved BEL cells were treated with the indicated drugs starting 1 h before addition of PTN (10 ng/ml), bFGF (1 ng/ml), or 5% FCS for another 18 h. Inhibitors used: A, orthovanadate (12.5 μM), genistein (2 μg/ml), pertussis toxin (1 μg/ml), or tyrphostin A25. The background for the right panel (without FCS) was 4192 ± 392 cpm. B, MEK-1 inhibitor PD98059. C, PI 3-kinase inhibitor wortmannin (10 μM). For details see "Materials and Methods."
DISCUSSION

PTN is a heparin-binding growth factor that is involved in cell growth and differentiation processes. It stimulates neurite outgrowth, fibroblast, endothelial, and epithelial cell growth (reviewed in Ref. 4). We report here a role of MAP kinase and PI 3-kinase pathways in the mitogenic activity of PTN. Experiments carried out in the presence of signal transduction inhibitors showed that drugs interfering with the tyrosine kinase pathway, genistein and tyrphostin A25, inhibited the mitogenic effect of PTN whereas an inhibitor of the G-protein coupled pathway, pertussis toxin, did not. This result demonstrates that PTN stimulates cell division through a tyrosine kinase pathway and that the PTN receptor is not a G protein-coupled receptor. Interestingly, the different inhibitors showed a different sensitivity of the PTN- versus the bFGF-induced DNA synthesis which indicates to us a differential sensitivity either of the respective receptor tyrosine kinases or of their coupling to the pathway downstream. Furthermore, we also found two novel, yet unidentified 190- and 215-kDa phosphoproteins in the lysates found that the MEK1 inhibitor PD98059 as well as the PI 3-kinase inhibitor wortmannin (10 nM; 1 h) were separated by SDS-PAGE (10% gel) and tyrosine-phosphorylated proteins present in the lysates were detected by Western blotting with the 4G10 anti-phosphotyrosine antibody (WB PY). Both panels represent blots from the same gel transferred for 2 h (bottom) and then overnight (top) using a second nitrocellulose membrane. SHC proteins on the membrane in the bottom panel were identified after stripping and reprobing with an anti-SHC antibody (WB SHC).

Activation of the PI 3-Kinase Pathway following PTN Stimulation—Since wortmannin inhibited PTN-induced mitogenesis, we decided to also assay for PI 3-kinase activation and used an in vitro assay of the Akt1 kinase to address that. Akt1 (protein kinase B) has been previously shown to be a substrate of PI 3-kinase and to be activated by phosphorylation (21). For our studies, Akt1 was immunoprecipitated from lysates of unstimulated or stimulated cells and immunoprecipitates were used for in vitro phosphorylation of myelin basic protein. The data presented in Fig. 7 show an increase of phosphorylation of myelin basic protein following PTN stimulation and a reversal by pretreatment with 10 nM wortmannin. These results indicate that Akt1 activation by PTN was mediated by PI 3-kinase. We conclude from this that PTN also activates the PI 3-kinase pathway to exert its mitogenic effect.

FIG. 4. PTN-induced tyrosine phosphorylation of 190- and 215-kDa proteins. Top panel, cell lysates from control (lanes 1 and 3) or PTN-stimulated cells (10 ng/ml for 10 min; lanes 2 and 4) were subjected to direct electrophoresis and Western blotting (WB PY; lanes 1 and 2; 25 μg of lysate per lane) or to immunoprecipitation and subsequent Western blotting (IP PY, WB PY; lanes 3 and 4; 500 μg of lysate) with the anti-phosphotyrosine antibody 4G10. For immunoprecipitations, agarose-coupled 4G10 antibody was used. Details under “Materials and Methods.” Bottom panel, in a parallel experiment, cell lysates (25 μg) from control cells (lane 1), PTN-stimulated cells (10 ng/ml, 10 min; lane 2), or bFGF-stimulated cells (100 ng/ml, 10 min; lane 3) were subjected to electrophoresis and Western blotting with the 4G10 antibody.

FIG. 5. PTN-induced tyrosine phosphorylation of SHC, ERK1, and ERK2. Cell lysate (25 μg) from control cells (lane 1) or from cells stimulated with PTN (10 ng/ml; 10 min) without (lane 2) and with pretreatment with the PI 3-kinase inhibitor wortmannin (10 nM; 1 h) were separated by SDS-PAGE (10% gel) and tyrosine-phosphorylated proteins present in the lysates were detected by Western blotting with the 4G10 anti-phosphotyrosine antibody (WB PY). Both panels represent blots from the same gel transferred for 2 h (bottom) and then overnight (top) using a second nitrocellulose membrane. SHC proteins on the membrane in the bottom panel were identified after stripping and reprobing with an anti-SHC antibody (WB SHC).

FIG. 6. PTN-induced mobility shift of ERK1 and ERK2. Cell lysate (25 μg) from control cells (lane 1) or from cells stimulated with PTN (10 ng/ml; 10 min) without (lane 2) and with pretreatment with the PI 3-kinase inhibitor wortmannin (10 nM; 1 h) were separated by SDS-PAGE (10% gel) and ERKs present in the lysates were detected by Western blotting with a panERK antibody (see “Materials and Methods”). Phosphorylated (pERK) and non-phosphorylated (ERK) ERKs are indicated.
exerts its mitogenic effect through both the MAP kinase and the PI 3-kinase pathways. MEK1 is a component of the MAP kinase pathway which is downstream of activated receptor tyrosine kinases (23, 24). Intermediary steps involve the docking and phosphorylation of adaptor proteins (25) and we demonstrate here that the adaptor protein SHC becomes phosphorylated upon stimulation of BEL cells by PTN (Fig. 5, bottom panel). Activated SHC has been shown to bind the complex Grb2 (growth factor receptor-bound 2) and SOS (son of sevenless) which activates Ras by GTP exchange. Ras activates Raf, which in turn activates MEK1. The next step in the cascade is the activation by phosphorylation on tyrosine, serine, and threonine of the MAP kinases ERK1 and ERK2 by MEK1 which culminates in activation of several transcription factors (26). Following stimulation by PTN, ERK1 and ERK2 were indeed phosphorylated as demonstrated by Western blotting and the mobility shift of the proteins (Figs. 5 and 6). Furthermore, tyrosine kinase and MEK1 inhibitors blocked PTN-induced mitogenesis (Fig. 3, A and B), suggesting that the activation of the above signaling molecules was crucial for the growth factor activity of PTN.

Finally, we studied the participation of PI 3-kinase in the PTN signaling. PI 3-kinase is a heterodimeric protein kinase formed by two subunits of 85 and 110 kDa (27). The p85 subunit is a regulatory protein that mediates, via its SH2 domains, binding of PI 3-kinase to activated receptor tyrosine kinases. The p110 subunit possesses the catalytic domain of the protein. Following binding to a receptor tyrosine kinase, PI 3-kinase phosphorylates phosphoinositides which serve as second messengers in the activation of downstream kinases. Two pathways diverge from PI 3-kinase: one leads to phosphorylation of the p70S6K ribosomal subunit and the other one to activation of the Akt1 and Akt2 proteins (21, 28). Recent studies show that antisense Akt2 RNA inhibited tumor growth of transfected pancreatic cancer cells linking the PI 3-kinase pathway to tumor growth (29). Furthermore, PI 3-kinase and Akt were implicated in the prevention of apoptosis (reviewed in Ref. 30). Our results show that stimulation of cells with PTN leads to activation of Akt1 (Fig. 7) and this activation as well as mitogenesis of PTN was inhibited by the PI 3-kinase inhibitor wortmannin (Figs. 3C and 7). Taken together, these results show that the PI 3-kinase pathway is important for the mitogenic signaling of PTN. It is important to note that pretreatment of cells with wortmannin did not affect tyrosine phosphorylation of proteins involved in the Ras/Raf/ MAP kinase pathway, indicating that it functions independently from the PI 3-kinase pathway and that both pathways are essential for a full mitogenic signal.

Acknowledgment—We thank Joannah Hackenbruck for help in some of the experiments.

REFERENCES
1. Li Y. S., Münzer, P. G., Chauhan, A. K., Watson, M. A., Hoffman, R. M., Kedder, C. M., Milbrandt, J., and Deuel, T. F. (1999) Science 250, 1690–1694
2. Kadomatsu, K., Tomomura, M., and Muramatsu, T. (1988) Biochem. Biophys. Res. Commun. 151, 3121–3128
3. Raulais, D., Lagente-Chévallier, O., Guettet, C., Duprez, D., Courtois, Y., and Vigny, M. (1991) Biochem. Biophys. Res. Commun. 174, 708–715
4. Schulte, A. M., and Wellstein, A. (1997) in Tumour Angiogenesis (Lewis, C. M., Bicknell, R., and Ferrara N., eds) pp. 273–289, Oxford University Press, Oxford
5. Kojima, S., Inui, T., Muramatsu, H., Kimura, T., Sakakibara, S., and Muramatsu, T. (1995) Biochem. Biophys. Res. Commun. 216, 574–581
6. Basilico, C., and Moscatelli, D. (1992) Adv. Cancer Res. 59, 115–164
7. Hampton, B. S., Marshall, D. R., and Burgess, W. S. (1992) Mol. Biol. Cell 3, 85–93
8. Takamatsu, H., Itoh, M., Kimura, M., Gospodarowicz, D., and Amann, E. (1992) Biochem. Biophys. Res. Commun. 185, 224–230
9. Raulo, E., Julkunen, I., Merenmies, J., Pihlaskari, R., and Rauvala, H. (1992) J. Biol. Chem. 267, 14081–14141
10. Courty, J., Dauchel, M. C., Caruelle, D., Perderiset, M., and Barritault, D. (1992) Biochem. Biophys. Res. Commun. 180, 145–151
11. Fang, W., Hartmann, N., Chow, D. T., Riegel, A. T., and Wellstein, A. (1992) J. Biol. Chem. 267, 25889–25897
12. Delbe, J., Vacherot, F., Laarooubi, K., Barritault, D., and Courty, J. (1995) J. Cell. Physiol. 164, 47–54
13. Laarooubi, K., Delbe, J., Vacherot, F., Desgranges, P., Tardieu, M., Jaye, M., Barritault, D., and Courty, J. (1994) Growth Factors 10, 89–98
14. Chauhan, A. K., Li, Y. C., and Deuel, T. F. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 679–682
15. Crusharko, F., Riegel, A. T., and Wellstein, A. (1994) J. Biol. Chem. 269, 21358–21363
16. Crusharko, F., Schulte, A. M., Berchem, G. J., and Wellstein, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14753–14758
17. Schulte, A. M., Lai, S., Kurtz, A., Crusharko, F., Riegel, A. T., and Wellstein, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14769–14774
18. Kohn, A. D., Kovacina, K. S., and Roth, R. A. (1995) EMBO J. 14, 4288–4295
19. Wellstein, A., Lupu, R., Zunguñezier, G., Flamm, S. L., Cheville, A. L., Delli Bevi, P., Basilico, C., Lippman, M. E., and Kern, F. G. (1990) Cell Growth Differ. 1, 63–71
20. Schlessinger, J., and Ullrich, A. (1992) Neuron 8, 383–391
21. Franke, T. F., Yang, S., Chauhan, A. K., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R., and Testa, J. R. (1995) Cell 81, 727–736
22. Li, Y. S., and Deuel, T. F. (1993) Biochem. Biophys. Res. Commun. 195, 1089–1095
23. Seger, R., and Krebs, E. G. (1995) FASEB J. 9, 726–735
24. Heldin, C. H. (1995) Cell 80, 213–223
25. Pawson, T. (1995) Nature London 373, 573–580
26. Hill, C. S., and Treisman, R. (1995) Cell 80, 199–211
27. Dhand, R., Haru, I., Hiles, B., Gout, I., Panayotou, G., Fry, M. J., Yonemizu, K., Kasuga, M., and Waterfield, M. D. (1994) EMBO J. 13, 511–521
28. Burgher, B. M. T., and Coffer, P. J. (1995) Nature 376, 599–602
29. Chauhan, A. K., Ruggeri, R., Klein, W. M., Smoda, G., Aitomare, D. A., Watson, D. K., and Testa, J. R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 3636–3641
30. Franke, T. F., Kaplan, D. R., and Cantley, L. C. (1997) Cell 88, 435–437