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Physical and Functional Interactions between Protein Tyrosine Phosphatase α, PI 3-Kinase, and PKCδ

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The somatostatin analogue, TT-232 inhibits cell proliferation and induces apoptosis in a variety of tumor cells both *in vivo* and *in vitro*. While the early transient activation of Erk/MAPK was found to be important for the induction of cell cycle arrest, the signaling pathway leading to the activation of Erk/MAPK had not been fully established. Here we present evidence that activation of the Erk/MAPK pathway by TT-232 involves PI 3-kinase, PKCδ and the protein tyrosine phosphatase α (PTPα). We show a physical interaction of PI 3-kinase and PKCδ with PTPα and show that the tyrosine phosphatase plays a role in the activation of MAPK. In this process, PTPα Ser-180 and Ser-204 phosphorylation is critical for the induction of phosphatase activity, which is required for dephosphorylation of pp60$^{c\text{-}src}$. Taken together, we demonstrate the physical and functional association between PI 3-kinase, PKCδ and PTPα in a signaling complex that mediates the antitumor activity of the somatostatin analogue TT-232.

Somatostatin is a natural tetradecapeptide that was originally discovered and characterized as a hypothalamic inhibitor of pituitary growth hormone release (1). It inhibits secretion of insulin, glucagon, gastrin and secretin (2), and has also been identified as an important endogenous antiproliferative agent (3–5). To understand the molecular events leading to the inhibition of cell proliferation, the signaling events activated by somatostatin and its analogues have already been investigated. It has been shown that these peptides cause rapid stimulation of tyrosine phosphatases (PTPs), and induce the dephosphorylation of phosphorylated epidermal growth factor receptor (EGFR; 3, 5). Moreover, recent reports pointed out that somatostatin can inhibit cell proliferation via the activation of tyrosine phosphatases and the induction of cyclin-dependent kinase inhibitors (CKI) p21$^{Cip1/Waf1}$ and p27^{Zip1} (6, 7). Furthermore, somatostatin was found to induce apoptosis in a variety of tumor cell lines (8, 9).

TT-232 is a somatostatin analogue with a cyclopenta-ring structure: D-Phe-Cys-Tyr-D-Thr-Lys-Cys-Thr-NH_2. It causes a strong antiproliferative effect both *in vivo* and *in vitro* and induced apoptosis in tumor cells (10–13). Unlike somatostatin, TT-232 did not inhibit growth hormone release or gastrin secretion *in vivo*. Due to its antitumor effects, TT-232 is presently under phase II clinical trials as a drug against various types of cancer.

The binding of TT-232 to somatostatin receptor subtypes 1 and 5 has been shown *in vitro* (Jiang, J., unpublished results) suggesting a receptor mediated signaling mechanism. We could show that several elements are common in TT-232 and somatostatin signaling through SSTR 1. These include the rapid activation of protein tyrosine phosphatases (14) and the induction of cell cycle arrest. This antiproliferative activity is mediated through a signaling pathway involving PKCδ, c-Src kinase and MAP-kinase (15).

PTPα is a ubiquitously expressed receptor-like protein tyrosine phosphatase (RPTP) that has a single membrane spanning region and two cytoplasmic catalytic PTP domains (16). The cytoplasmic part of PTPα is subjected to both tyrosine phosphorylation (17) and serine phosphorylation upon stimulation with the PKC activator TPA (18). The extracellular part of PTPα is heavily glycosylated but, unlike other RPTPs involved in cell-cell or cell-matrix adhesion events, PTPα lacks immunoglobulin-like or type III fibronectin domains. A functional ligand of PTPα has not yet been identified, which suggests that intracellular signaling pathways significantly contribute to the regulation of PTPα (19). Previously identified substrates for PTPα include several proteins of the c-Src family kinases (20, 21) and the insulin receptor (22, 23).

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In this report, we investigated the activation of c-Src by PKCδ in the antiproliferative signaling mechanism of TT-232, and we show the involvement of PTPα. We also present evidence that TT-232 induces the physical and functional interaction between PI 3-kinase, PTPα and PKCδ. We demonstrate that the activation of PI 3-kinase and PKCs lead to the stimulation of PTPα activity and the activation of a downstream signaling pathway through phosphorylation of Ser-180 and Ser-204 of the phosphatase. This results in dephosphorylation of c-Src. Our findings expand the current signaling model for the antiproliferative somatostatin analogue TT-232 and suggest a general interaction mechanism of PTPα, PKCδ and PI 3-kinase.

MATERIALS AND METHODS

Reagents, antibodies, and plasmids. Antibodies purchased were mouse monoclonal anti-PI 3-kinase (clone 93-3, Upstate Biotechnology Inc., Lake Placid, NY), mouse monoclonal anti-c-Src (Upstate Biotechnology Inc., Lake Placid, NY), monoclonal anti-HA antibody 12CA5 (Roche, Mannheim, Germany), mouse monoclonal anti-PKCδ (Transduction Laboratories, Lexington, KY), mouse monoclonal anti-phosphotyrosine (clone 4G10, Upstate Biotechnology Inc.). Polyclonal PTPα antibody and polyclonal p110β antibody were generated in our laboratory (23). Respective secondary antibodies were obtained form Bio-Rad (Hercules, CA) and Dianova (Hamburg, Germany). For immunoblot detection, the ECL system (Amersham, Uppsala, Sweden) was used. Protein-A Sepharose was from Pharmacia Biotech. Inc. (Uppsala, Sweden). All other reagents were from Sigma (St. Louis, MO).

All cDNAs used were in CMV promoter driven expression plasmids. Mouse Erk2 cDNA (24) was fused with an N-terminal hemagglutinin (HA) epitope as described by Meloche *et al.* (25). Catalytically inactive human PTPα where both cysteines (Cys442 and Cys732) (23) were changed to serines and serine to alanine mutant cDNAs were generated by *in vitro* mutagenesis. TT-232 was synthesized as described earlier (11, 12). For treatments, a 2 mg/ml stock solution dissolved in water was used.

Cell culture and transfections. A431 and Cos-7 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Cos-7 cells were transiently transfected using Lipofectamine Plus (Gibco-BRL, Rockville, MD). Transfections were made in 6-well dishes according to the manufacturer's instructions. The transfection mixtures were replaced by 10% fetal calf serum containing medium after 4 h, and 20 h later, cells were washed and starved for 24 h in serum-free medium.

Cell lysis, immunoprecipitation, and immunobloting. Prior to lysis, cells grown to 70% confluency were starved for 24 h and treated with inhibitors and agonists as indicated, washed once with PBS and lysed for 5 min on ice in buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium-pyrophosphate, 2 mM sodium-vanadate, 10 mM sodium-fluoride, 1 mM PMSF, 10 μg/ml aprotinin and 2 μg/ml leupeptin. Lysates were cleaned by centrifugation at 13,000 rpm (10,000*g*) for 5 min at 4°C and cell lysates were precleaned with 2 μg non-immune serum and 30 μl protein A-Sepharose for 2 h at 4°C. Supernatants were used for immunoprecipitation using the respective antibodies and 30 μl protein A-Sepharose for 4–10 h at 4°C. Precipitates were washed three times with 0.5 ml of HNTG buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) (26), suspended in SDS sample buffer, boiled, subjected to gel electrophoresis and transferred to nitrocellulose in a semi-dry blotting apparatus with 0.8 mA/cm^2 current for 2 h.

PI 3-kinase assay. Endogenous PI 3-kinase was immunoprecipitated from lysates obtained from 10 cm dishes using 3 μg antibody. Immunoprecipitates were washed twice with 0.75 ml of HNTG buffer (26) and three times with 0.75 ml of Triton extraction buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.2 mM sodium-vanadate). Kinase reactions were performed in 50 μl of kinase reaction buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 200 μM adenosine, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 20 μM ATP, 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol)), supplemented with sonified 100 μM phosphatidyl-inositol, 200 μM phosphatidyl-serine in 20 mM Tris–HCl, pH 7.5 for 30 min at 30°C. Reactions were stopped with 50 μl 1M HCl, phosphorylated lipid products were extracted with organic extraction and subjected to TLC. The solvent used was a mixture of chloroform/methanol/4 M NH_4OH (9:7:2 by volume).

MAPK assay. Epitope-tagged HA-Erk2 was immunoprecipitated from lysates obtained from 6-well dishes using 2.5 μg of 12CA5 antibody. Immunoprecipitates were washed three times with 0.25 ml of HNTG buffer (26) and once with 0.4 ml of MAPK kinase buffer (containing 20 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT and 200 μM sodium-vanadate). Subsequently, kinase reactions were performed in 30 μl of MAPK kinase buffer supplemented with 0.5 mg/ml myelin basic protein, 50 μM ATP and 1 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) for 10 min at room temperature. Reactions were stopped and subjected to gel electrophoresis on 15% polyacrylamide gels. Radioactivity was quantified using a Phosphoimager (Fuji).

Preparation of A431 membranes. A431 cells were washed once with buffer A (10 mM sodium-phosphate pH 7.0, 150 mM NaCl) and scraped. Cells were collected by centrifugation, resuspended in buffer B (50 mM Tris–HCl, pH 7.0, 1 mM PMSF, 10 μg/ml aprotinin and 2 μg/ml leupeptin) and lysed by freeze thawing in liquid nitrogen. The lysate was centrifuged at 170*g* for 5 min, the supernatant was subsequently centrifuged at 25,000*g* for 30 min. The pellet was resuspended in 200 μl buffer B.

Preparation of phosphorylated poly (Glu, Tyr). Phosphorylated poly (Glu, Tyr) (4:1) was prepared by incubating 2 mg polymer with A431 cell membranes containing 200–500 μg protein in 50 mM Hepes, pH 7.4, 0.1% Triton-X 100, 15 mM $MgCl_2$, 240 μM [γ-^{32}P]ATP, 100 μM ATP, 100 μM sodium-vanadate at 4°C overnight. Substrate was precipitated with TCA and dissolved in PBS.

Assay for PTPα activity. Prior lysis, cells grown to 90% confluence were starved for 24 h, treated with ligands and inhibitors as indicated, washed once with PBS and lysed for 5 min on ice in buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium-fluoride, 1 mM PMSF, 10 μg/ml aprotinin and 2 μg/ml leupeptin. Lysates were precleaned by centrifugation at 13,000 rpm (10,000*g*) for 5 min at 4°C. PTPα was immunoprecipitated from cell lysates obtained from 10 cm dishes using 4 μg of polyclonal anti-PTPα antibody. Immunoprecipitates were washed three times with 0.25 ml of HNTG buffer (26) and once with 0.5 ml of phosphatase buffer (50 mM Tris–HCl, pH 7.5, 10 mM DTT, 2 mM EDTA). Phosphatase reactions were performed in 50 μl of phosphatase buffer supplemented with 100,000 cpm of [^{32}P]-labeled poly (Glu, Tyr) as substrate for 5 min at 30°C. The inorganic [^{32}P]-phosphate liberated was extracted using the molybdate extraction procedure (27) and radioactivity was evaluated by liquid scintillation.

Phosphoamino-acid analysis. Phosphoamino-acid analysis was carried out as described previously (28). Briefly, following gel electrophoresis and transfer onto PVDF membranes excised proteins were subjected to acid hydrolysis in 6 M HCl for 1 h. The hydrolyzed products were spotted on cellulose thin-layer chromatography plates together with a non-labeled phosphoamino-acid standard mix. Samples were separated by two-dimensional thin-layer chromatography at pH 1.9, and pH 3.5 for the first, and second dimension, respectively. Phosphoamino-acid standards were visualized with 0.25% ninhydrine in acetone, and radiolabeled products were detected by autoradiography.

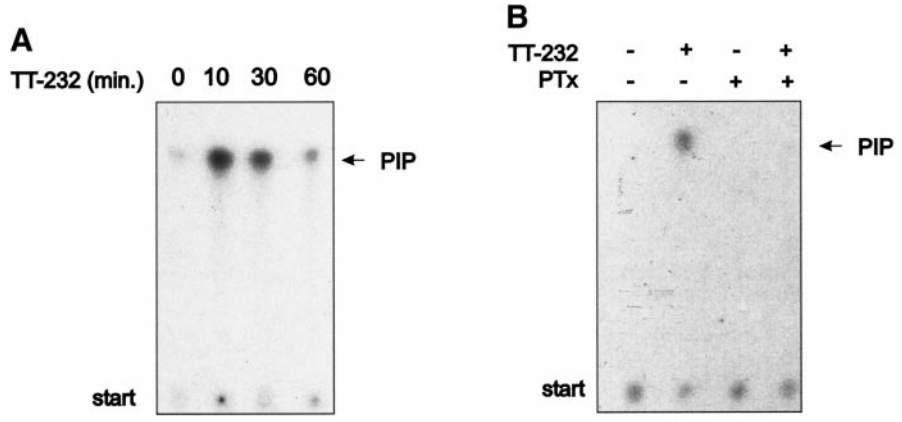

FIG. 1. TT-232 induces PI 3-kinase activity in a $G_{i/0}$ protein dependent manner. Serum starved A431 cells were treated for the indicated times with 50 μM TT-232. Immunoprecipitated endogenous PI 3-kinase was subjected to *in vitro* kinase assays, and phosphorylated lipid products were visualized by autoradiography after TLC separation. To investigate the role of $G_{i/0}$ proteins, A431 cells were pretreated with 100 ng/ml pertussis toxin (PTx) for 16 h and induced with 50 μM TT-232 for 10 min.

RESULTS AND DISCUSSION

TT-232 Induces PI 3-Kinase Activity through a Pertussis Toxin Sensitive G-Protein

Our previous results showed that the activation of ERK/MAPK pathway by TT-232 is wortmannin sensitive (15). Based on these findings we analyzed the effect of TT-232 on the activity of PI 3-kinase in *in vitro* kinase assay. In A431 cervix carcinoma cells TT-232 induced PI 3-kinase activity after 10 min (Fig. 1A), which declined back to control levels after 60 minutes. Preincubation of A431 cells with 100 ng/ml pertussis toxin completely abolished the induction of PI 3-kinase (Fig. 1B). These results demonstrate that PI 3-kinase activity is induced transiently in a $G_{i/0}$-protein dependent manner upon TT-232 treatment, extending our previous demonstration of a critical involvement of PI 3-kinase activity in the downstream signaling and the biological effect of TT-232 (15).

Physical Interaction between PI3-Kinase, PTPα, and PKCδ

The protein tyrosine-phosphatase PTPα was shown to be phosphorylated and activated by PKCs (18) and to regulate the activity of c-Src family members. We previously demonstrated that PKCδ and c-Src are involved in the activation of ERK/MAPK and cell cycle arrest by TT-232 (15). These results suggested that PTPα might be involved upstream of c-Src triggering the ERK/MAPK upon treatment with TT-232.

To support this hypothesis, we investigated the physical interaction of PI-3 kinase and PKCδ with PTPα. The phosphatase was immunoprecipitated from A431 cell lysates, blotted and probed with an antibody targeted against the 110-kDa catalytic subunit of PI-3 kinase. A 110-kDa protein band was found to coprecipitate with PTPα following TT-232 treatment (Fig. 2A).

A reciprocal experiment was carried out to confirm the association of PTPα with PI-3 kinase. p110 was immunoprecipitated from A431 cells and analyzed for the presence of PTPα. Probing the membrane with PTPα antibody showed the association of the phosphatase and PI3-kinase in TT-232 treated cells (Fig. 2B).

To test the assumed presence of PKCδ in the complex, PTPα (Fig. 2C, upper panel) or PI3-kinase p110 (Fig. 2D, upper panel) immunoprecipitations were carried out and PKCδ was detected with monoclonal antibody only in TT-232 treated cells.

Co-immunoprecipitation experiments with endogenously expressed PI 3-kinase and PTPα proteins have shown their association together with PKCδ upon treatment with the somatostatin analogue. This indicates the formation of a signaling complex upon treatment and suggests a functional interaction between the molecules. Our findings are supported by the association of PI 3-kinase and PKCδ, which has been found upon cytokine stimulation (29) suggesting an analogous pathway. However, the protein domains involved in the interaction needs further investigations.

The Association of PTPa and PKCδ Does Not Require the Activity of PI3-Kinase

To test the requirement of PI 3-kinase activity for the formation of the signaling complex we pretreated A431 cells with 100 nM wortmannin or 1 μM Ly294002 for 15 min prior to treatment with 50 μM TT-232 for 10 min. Figure 3 demonstrates that the formation of the signaling complex between PKCδ, PTPα and PI 3-kinase does not require the catalytic activity of PI 3-kinase. However, the activation of downstream signals does require PI 3-kinase activity. This can be

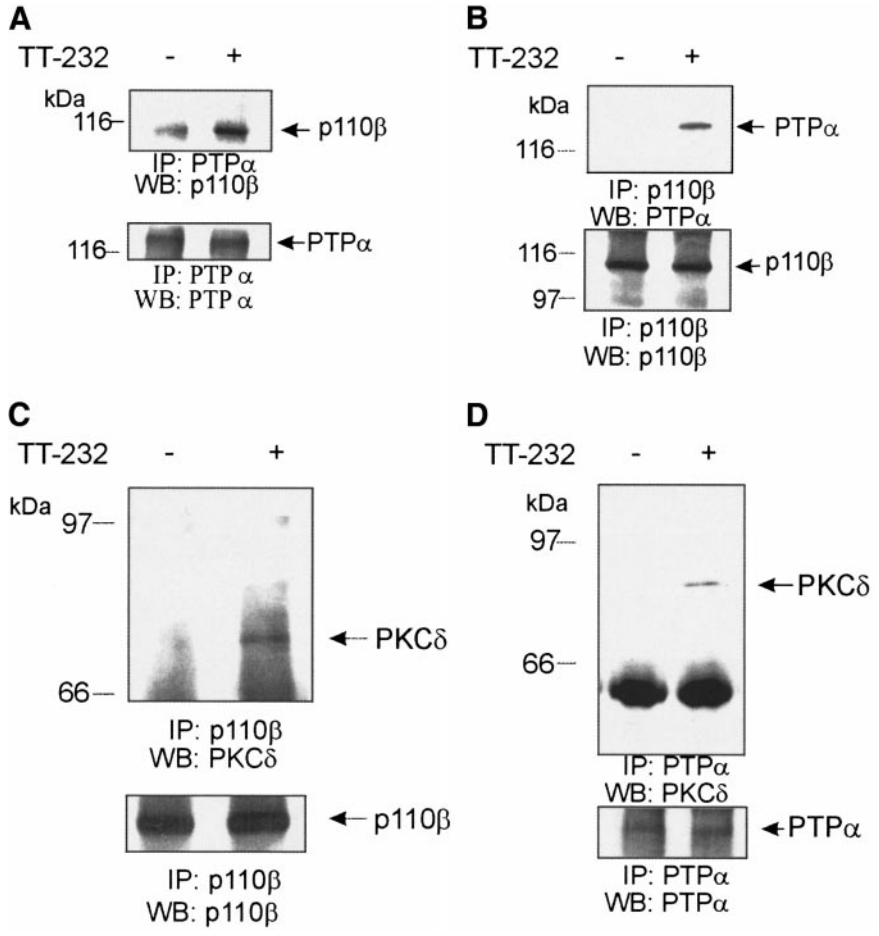

FIG. 2. TT-232 induced association of PI 3-kinase, PKCδ and PTPα. (A) Starved A431 cells were stimulated with 50 μM TT-232 for 10 min prior lysis. Cells lysates were subjected to immunoprecipitation with PTPα polyclonal antibody. Following polyacrylamide gel electrophoresis and transfer to nitro-cellulose, membrane was probed for the presence of the p110β catalytic subunit of PI 3-kinase (upper panel). The amount of PTPα was analyzed by reprobing the filter with anti-PTPα polyclonal antibody (lower panel). (B) Reciprocal experiments were carried out from A431 cell lysates. Endogenous p110β was immunoprecipitated and probed with PTPα antibody (upper panel). The amount of p110β was analyzed by reprobing the filter with anti-p110β polyclonal antibody (lower panel) (C) A431 cells were stimulated with 50 μM TT-232 for 10 min and cell lysates were subjected to immunoprecipitation with PTPα polyclonal antibody. Membrane was probed for the presence of PKCδ (upper panel). The amount of PTPα was analyzed by reprobing the filter with anti-PTPα polyclonal antibody (lower panel). (D) Starved A431 cells were treated with 50 μM TT-232 for 10 min. Cells were lysed and p110β catalytic subunit of PI 3-kinase was immunoprecipitated. Polyacrylamide gel electrophoresis and transfer to nitrocellulose was performed and the membrane was probed for PKCδ (upper panel). Equal loading was shown by reprobing the filter with anti-p110β polyclonal antibody (lower panel).

explained by the involvement of yet unidentified proteins upstream of PI 3-kinase.

PTPα Lies Downstream of PI 3-Kinase and PKCδ and Is Required for the Induction of Erk/MAPK

We investigated the role of PI 3-kinase and PKC inhibitors on PTPα activity. Cells were pretreated either with 100 nM wortmannin, or with 2.5 μM GF109203X (30) prior to treatment with TT-232. Figure 4A shows that TT-232 increased PTPα activity up to 2.5 fold over basal level and both PI-3 kinase and PKC inhibitors abolished this effect. These findings place PTPα downstream of PI 3-kinase and PKCδ in TT-232 signaling.

To study downstream events of PTPα activation, Cos-7 cells were transiently transfected with either wild type or a catalytically inactive mutant form of PTPα (C442/732S) together with hemagglutinin-tagged Erk2. The activity of Erk/MAPK was measured from anti-hemagglutinin immunoprecipitates (Fig. 4B). The expression of the inactive form of PTPα completely abolished TT-232-induced Erk/MAPK activation. This demonstrates that the already reported activation of Erk/MAPK by TT-232 (15) is dependent on the function of PTPα.

It has been shown that PKCs phosphorylate PTPα on Ser-180 and Ser-204 *in vitro* and *in vivo* upon TPA treatment and thereby induce the activity of the phos-

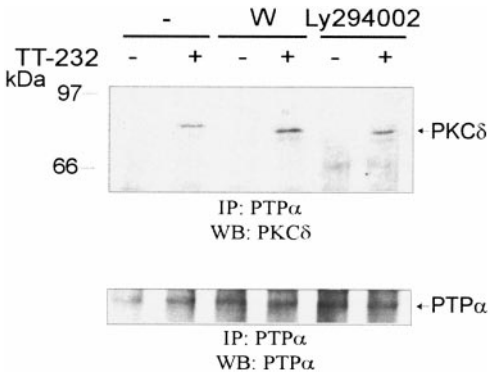

FIG. 3. PI 3-kinase activity is not required for the association of PTPα and PKCδ. A431 cells were pretreated with vehicle, 100 nM wortmannin or 1 μM Ly294002 respectively, for 15 min prior stimulation with 50 μM TT-232 for 10 min. Endogenous PTPα was immunoprecipitated with PTPα polyclonal antibody. Following polyacrylamide gel electrophoresis proteins were transferred to nitrocellulose and probed for the presence of the PKCδ (upper panel). The amount of PTPα was analyzed by reprobing the filter with anti-PTPα polyclonal antibody (lower panel).

phatase (18). Since we had observed the requirement of PKCs for the activation of PTPα by TT-232 (Fig. 4A), we tested the effect of PTPα Ser-180 and Ser-204 sites in the activation of Erk/MAPK in our system. Figure 4B shows that overexpression of the Ser180/204Ala double mutant of PTPα was sufficient to abrogate the ERK/MAPK stimulation induced by TT-232. This implies that TT-232 signaling employs the same mechanism in this part of its pathway as it has been described for TPA (18).

To investigate the phosphorylation of PTPα in detail, Cos-7 cells were transiently transfected with plasmids encoding wild type, catalytically inactive PTPα (Cys442/732Ser) or the Ser180/204Ala double mutant followed by [^{32}P]-*ortho*-phosphate labeling for 24 h. Cells were treated with 50 μM TT-232 for 10 min, PTPα was immunoprecipitated with a polyclonal anti-PTPα antibody, and the immunoprecipitates were subjected to phosphoamino-acid analysis (Figs. 5A–5F). Upon treatment with TT-232, serine phosphorylation of wild type PTPα is induced (Fig. 5B). The increased tyrosine phosphorylation of PTPα is in concurrence with previous results suggesting that phosphorylation of Tyr-789 of PTPα by c-Src provides a potential feedback loop (17).

TT-232 induced serine phosphorylation also of catalytically inactive PTPα (Figs. 5C and 5D) meaning that the activity of PTPα is not required for phosphorylation. The increased tyrosine phosphorylation compared to the wild type PTPα is likely to be due to the lack of the autocatalytic activity of PTPα as reported previously (17).

In cells overexpressing Ser180/204Ala double mutant PTPα, no significant increase in phosphoamino-acid content was detectable upon treatment with TT-232 (Figs. 5E and 5F). These results confirm the important role of serines 180 and 204 in the activation of PTPα, and are in agreement with the data of Tracy *et al.* (18) who demonstrated the same serines to be essential in the stimulation of PTPα by TPA.

The fact that phosphorylation of the Ser180/204 sites is crucial for the activation of the Erk/MAPK pathway

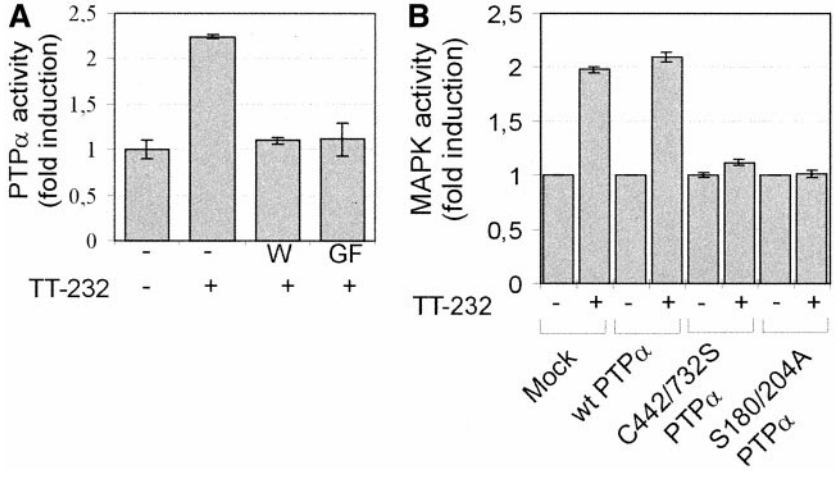

FIG. 4. (A) PTPα is activated by PKC. Following a starvation for 16 h, A431 cells were pre-treated with vehicle, 100 nM wortmannin or 25 μM GF109203X, respectively, for 15 min prior stimulation with 50 μM TT-232 for 10 min. Immunoprecipitated endogenous PTPα was subjected to an *in vitro* phosphatase assay in the presence of 100,000 cpm [^{32}P]-poly (Glu, Tyr) (4:1). After organic extraction released [^{32}P] was measured by scintillation counting. Data shown represent the means ± SD of three independent experiments. (B) PTPα activity and phosphorylation of Ser-180 and Ser-204 is required for the induction of its downstream signaling. Cos-7 cells were transfected either with the vector alone, or with plasmid DNA encoding the wild type, catalytically inactive or the double serine mutant form of PTPα respectively, with the addition of a plasmid encoding HA-Erk2. Cells were stimulated with 50 μM TT-232 for 10 min prior lysis. Immunoprecipitated HA-Erk2 was subjected to an *in vitro* kinase assay, and phosphorylated MBP was visualized by autoradiography after gel electrophoresis. Data shown represent the means ± SD of three independent experiments.

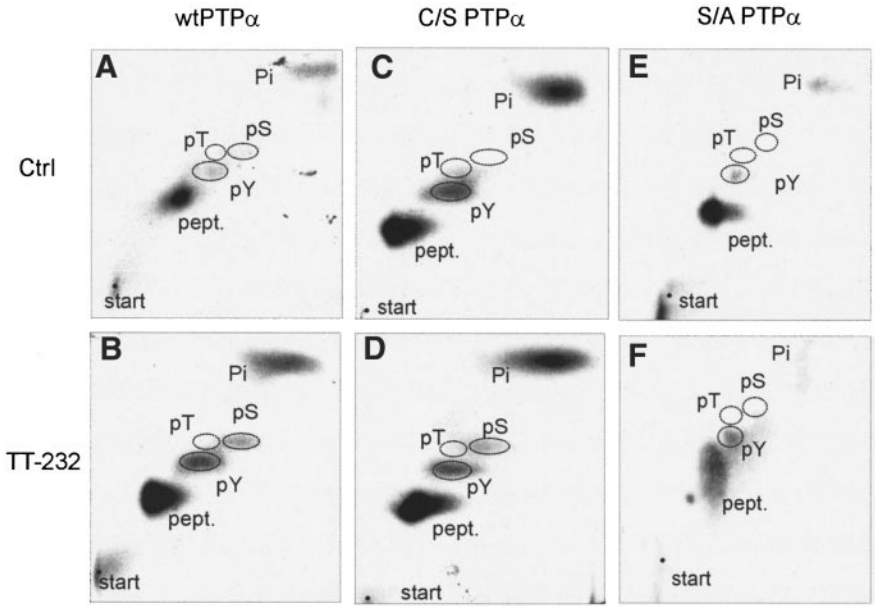

FIG. 5. TT-232 induces the phosphorylation of PTPα. Cos-7 cells were transiently transfected with plasmids encoding either wild type (A and B), catalytically inactive (PTPα C442/732S) (C and D), or Ser180/204Ala double mutant PTPα (E and F) cDNAs followed by [^{32}P]*ortho*-phosphate labeling for 24 h. Cells were treated either with vehicle (A, C, and E) or with 50 μM TT-232 (B, D, and F) for 10 min, PTPα was immunoprecipitated with a polyclonal anti-PTPα antibody and the immunoprecipitates were subjected to phosphoamino-acid analysis.

by TT-232, together with the necessity of functional PKCs for PTPα activation in *in vitro* phosphatase experiments makes us propose that these serine sites are phosphorylated by the upstream protein PKCδ and not by other kinases.

Dephosphorylation of c-Src by PTPα

In PTPα-overexpressing fibroblasts, c-Src kinase is dephosphorylated at its C-terminal regulatory Tyr-527 site, which increases the activity of the kinase through the direct action of PTPα (20, 31). Since we were able to demonstrate that TT-232 leads to the activation of c-Src (15), the phosphotyrosine content of c-Src upon stimulation with TT-232 was investigated in Cos-7 cells overexpressing either the wild type, or the catalytically inactive Cys442/732Ser mutant of PTPα.

Cells were maintained in serum free medium for 16 hours prior to immunoprecipitation with c-Src antibodies. Immunoprecipitates were blotted with phosphotyrosine antibody, which revealed a double band corresponding to different phosphorylation states of c-Src: a higher band migrating at ~62 kDa, consistent with the stronger phosphorylated form of c-Src, and a lower band at ~60 kDa, likely to be corresponding to weaker phosphorylated c-Src protein. In wild type PTPα overexpressing Cos-7 cells, treatment with TT-232 leads to reduced intensity of the upper band, which means that c-Src is dephosphorylated. In cells carrying catalytically inactive PTPα this effect was blocked. Therefore, the phosphatase activity of PTPα is required for c-Src dephosphorylation likely by direct interaction (Fig. 6A). To support the results, Cos-7 cells were transiently transfected either with wild type or catalytically inactive Cys442/732Ser PTPα together with c-Src followed by [^{32}P]-ortho-phosphate labeling. Cells were treated with 50 μM TT-232 for 10 min, c-Src was immunoprecipitated and the immunoprecipitates were subjected to phosphoamino-acid analysis as described above. Treatment with TT-232 resulted in dephosphorylation of c-Src tyrosine residues but did not affect the phosphorylation state of serine or threonine amino acids (Figs. 6B and 6D). However, in cells overexpressing catalytically inactive PTPα dephosphorylation of tyrosine upon TT-232 treatment was not visible and the phosphoamino-acid pattern was similar to that of the untreated cells (Figs. 6C and 6E).

These observations strengthen our hypothesis that the catalytic activity of PTPα is required for the dephosphorylation and thus activation of c-Src upon treatment with the somatostatin analogue. They are concordant with the understanding of c-Src regulation by PTPα proposed by Zheng *et al.* (31). Therefore, this is likely that the activation of c-Src by dephosphorylation of the regulatory Tyr-527 site by PTPα is the mechanism of the activation of the MAPK pathway reported previously (15).

We propose a model where the treatment with TT-232 through somatostatin receptor 1 induces the formation of a signaling complex composed of PI 3-kinase, PTPα and PKCδ. The activation of PI 3-kinase through

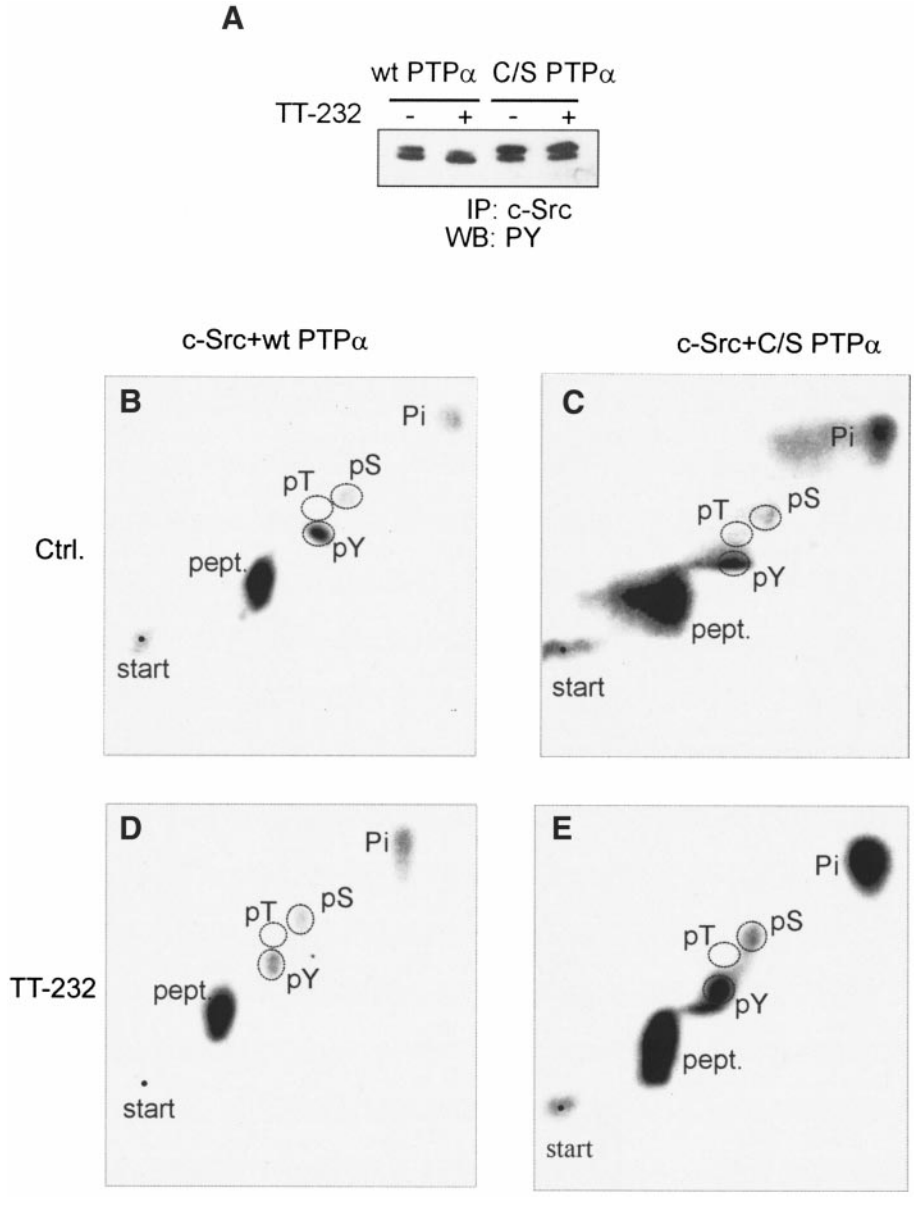

FIG. 6. TT-232 induces the dephosphorylation of tyrosine residues of c-Src by PTPα. (A) Cos-7 cells were transiently transfected with plasmids encoding either wild type or catalytically inactive (PTPα C442/732S) PTPα cDNAs. Cells were starved, treated with 50 μM TT-232 for 10 min and c-Src was immunoprecipitated. Following SDS-gel electrophoresis immunoprecipitates were blotted with anti-phosphotyrosine antibody. (B–E) Cos-7 cells were transiently transfected either with wild type, or catalytically inactive PTPα (PTPα C442/732S) together with c-Src followed by [^{32}P]-*ortho*-phosphate labeling. Cells were treated with 50 μM TT-232 and c-Src was immunoprecipitated. The radiolabeled bands were excised and subjected to phosphoamino-acid analysis as described above.

$G_{i/o}$ enhances PKCδ activity which in turn phosphorylates Ser180 and Ser204 of PTPα. This leads to the activation of the tyrosine phosphatase that dephosphorylates its major substrate c-Src at the inhibitory Tyr-527. The increased activity of c-Src mediates the signal to the Erk/MAPK pathway (Fig. 7). The activation of the proposed signaling cascade has been demonstrated to play a crucial role in the G_1-to-S phase arrest induced by TT-232 (15).

The results reported here expand our concept of the mechanism of Erk activation by TT-232. Since Erk

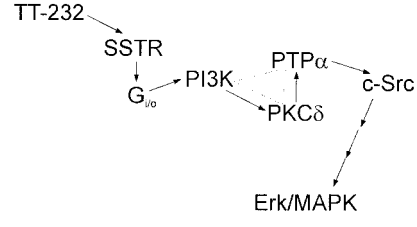

FIG. 7. Model of the TT-232 induced signaling complex. Gray box shows the physical interaction between the proteins.

activation seems to be required for cell cycle arrest induced by somatostatin and somatostatin analogues, we conclude that the formation of this signaling complex and its functional integrity is necessary for the observed biological effect. Apart from shedding light on the cellular events mediated by the anti-cancer drug TT-232, our results are of great interest to understand the signaling mechanisms of somatostatin and somatostatin analogues as well as of the regulation mechanisms of PKCs and PTPα.

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