Biochemical and Biophysical Research Communications **285,** 483–488 (2001)
doi:10.1006/bbrc.2001.5199, available online at http://www.idealibrary.com on IDEAL®

The Antitumor Somatostatin Analogue TT-232 Induces Cell Cycle Arrest through PKCδ and c-Src

Attila Steták,*[,1] Andreas Lankenau,† Tibor Vántus,* Péter Csermely,* Axel Ullrich,† and György Kéri*

**Peptide Chemistry Research Group, Department of Medical Chemistry, Semmelweis University, Budapest, H-1088 Hungary; and †Department of Molecular Biology, Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, D-82152 Martinsried, Germany*

Received May 31, 2001

The heptapeptide TT-232 is structurally related to the hypothalamic hormone somatostatin and shows promise as an anticancer drug because of its tumor-specific cytotoxic effects. Apart from the ability to induce apoptosis, the synthetic peptide can trigger an alternative pathway that leads to cell cycle arrest in certain tumor cell systems. We found that pulse treatment with TT-232 blocks the cell cycle G_1/S transition irreversibly in A431 cells. Investigation of the TT-232 signaling pathway yielded results similar to those reported for somatostatin although its affinity to the somatostatin receptor 1 is significantly reduced. We show that functional protein kinase C (PKC) δ as well as c-Src are necessary mediators of the TT-232 cytostatic effect and we propose a signaling pathway that leads to cell cycle arrest.

Key Words: **somatostatin analogue; MAPK; PKC delta; c-Src; cell cycle arrest; $p21^{Cip1/Waf1}$; tumor; cytostatic.**

Somatostatin is a natural tetradecapeptide that was originally discovered and characterized as a hypothalamic inhibitor of pituitary growth hormone release (1). Its physiological roles include the inhibition of insulin, glucagon, gastrin and secretin secretion (2), and it has also been described as an endogenous antiproliferative agent (3, 4). Several potent somatostatin analogues have been developed as antisecretory and antiproliferative agents such as Sandostatin, RC-160, and Somatuline (5).

The somatostatin structural analogue TT-232 is a heptapeptide of a cyclopenta-ring structure: D-Phe-Cys-Tyr-D-Thr-Lys-Cys-Thr-NH_2 that was shown to have strong antiproliferative and apoptotic effects on tumor cells both *in vivo* and *in vitro* (6–8) but did not inhibit growth hormone release or gastrin secretion *in vivo.* It is presently under phase II clinical trials as a drug against various types of cancer. The signaling mechanisms underlying its strong effects on tumor cells are, however, only poorly understood.

Somatostatin can bind to at least five known receptors, the $G_{i/o}$-protein coupled somatostatin receptors (SSTR 1–5), whose expression patterns vary amongst diverse cell types (9–10). It has been found that somatostatin can trigger different signaling pathways depending on the expressed SSTRs (11).

Unlike somatostatin, TT-232 binds to SSTR 1 and SSTR 5 (J. Jiang, unpublished results). Since SSTR5 was found to be expressed only in a minor fraction of examined tumor cell lines and tissues (12, 13), we decided to focus our investigations on SSTR1-related signaling events.

Upon somatostatin stimulation of ectopically expressed SSTR1 in CHO-K1 cells, Florio *et al.* (14) found decreased cell proliferation accompanied by activation of the ERK/MAPK pathway, which was dependent on the function of phospho-inositide-3-kinase (PI3K). We obtained similar results with TT-232 treatment of A431 and COS-7 cells which both express endogenous SSTR1. It has been demonstrated that PI3K is involved in the activation of protein kinase C (PKC) family members (15, 16). Since PKCδ activity was able to inhibit proliferation in several cell lines through cell cycle arrest (17–19), we asked whether PKCs and especially PKCδ play a role in the signaling of TT-232. FACS analysis revealed that the antiproliferative properties of TT-232 are caused by irreversible cell cycle arrest at G_1/S transition that required functional PKCs and MEK. We were able to show that PKCδ is activated and translocated to the cell membrane upon TT-232 treatment. Introduction of a dominant negative mutant revealed that PKCδ is positioned upstream of ERK in the TT-232 signaling pathway.

[1] To whom correspondence should be addressed. Fax: (+49 89) 8578-3777. E-mail: stetak@hotmail.com.

0006-291X/01 $35.00

MATERIALS AND METHODS

Tissue culture and transfection. A431 cells and COS-7 cells from ATCC (Manassas, VA) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. COS-7 cells were transiently transfected using Lipofectamine Plus according to the manufacturer's instructions (Gibco-BRL, Rockville, MD).

Cell lysis and immunoblotting. Ninety percent confluent cells were starved for 24 h, 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 precleaned by centrifugation at 13,000 rpm (10,000*g*) for 5 min at 4°C. Samples were boiled in SDS sample buffer subjected to gel electrophoresis and transferred to Nitrocellulose in a semi-dry blotting apparatus with 0.8 mA/cm^2 current for 2 h.

Northern blot. Five micrograms total RNA was separated by formaldehyde denaturing agarose gel electrophoresis and transferred to nitrocellulose. The membrane was probed with human SSTR 1 fragment (bp 510–975) as probe under stringent conditions.

ERK/MAPK assay. Epitope-tagged HA-ERK2 was immunoprecipitated from lysates using 12CA5 antibody recognizing the YPYDVPDYA epitope of influenza hemagglutinin protein (La Roche, Mannheim, Germany). Endogenous ERK2 was immunoprecipitated using a polyclonal anti-ERK2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were washed three times with 0.25 ml of HNTG buffer (20) 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 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, samples were subjected to gel electrophoresis on 15% polyacrylamide gels and were quantified using a Phospho-Imager (Fuji).

Membrane preparation. Cell membranes were prepared as described previously (21). Briefly, A431 cells were grown in 150-mm dishes to 90% confluency. Cells were washed twice with 5 ml buffer A (0.25 M sucrose, 1 mM EDTA, 20 mM Tricine, pH 7.8) and cells were collected by scraping in buffer A, homogenized with 20 strokes in a 2 ml Wheaton tissue grinder. Following centrifugation at 1000*g* for 10 min the postnuclear supernatant was layered on top of 30% Percoll in buffer A and centrifuged at 84000*g* for 30 min. The plasma membrane fraction was a visible band, which was collected with a Pasteur pipette.

c-Src kinase assay. c-Src was immunoprecipitated from cell lysates using anti-c-Src monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY). Immunoprecipitates were washed three times with 0.25 ml of HNTG buffer (20) and once with 0.4 ml of Src kinase buffer (containing 20 mM Hepes, pH 7.5, 1 mM $MnCl_2$, 1 mM DTT, and 200 μM sodium vanadate). Subsequently, kinase reactions were performed in 20 μl of Src kinase buffer supplemented with 10 μg acid denatured enolase, 50 μM ATP and 5 μCi of (γ-^{32}P)ATP (3000 Ci/mmol) for 10 min at 30°C. Reactions were stopped by addition of 20 μl of 2 times concentrated SDS sample buffer and samples were subjected to gel electrophoresis on 10% polyacrylamide gels and labeled enolase was quantified using a Phospho-Imager (Fuji).

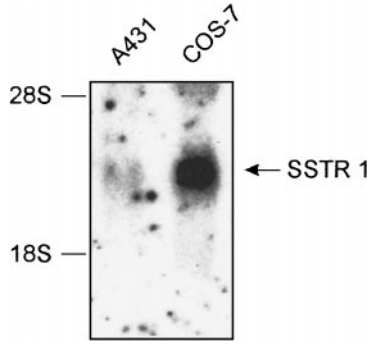

FIG. 1. Northern blot analysis of total RNA from A431 and COS-7 cells. SSTR1 is expressed in both cell lines at different levels.

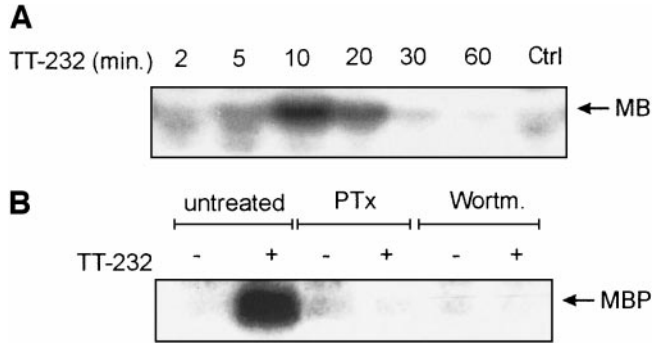

FIG. 2. Induction of ERK2 activity by TT-232. (A) A431 cells were treated with 50 μM TT-232 for the indicated times and lysed. Endogenous ERK2 was immunoprecipitated and incubated with (γ-^{32}P) ATP and myeloid basic protein (MBP) as substrate in *in vitro* kinase assay. Phosphorylated MBP was visualized by autoradiography after gel electrophoresis. (B) ERK activation by TT-232 was prevented by preincubation of the cells with 100 ng/ml pertussis-toxin (PTx) for 16 h, or 100 nM wortmannin (wortm.) for 15 min prior to treatment with 50 μM TT-232.

PKC kinase assay. The PKC activity was performed from immunoprecipitates with specific polyclonal PKCδ (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The kinase reaction was performed as described for MAPK assay in a reaction buffer supplemented with 50 μg/ml phosphoserine and histone H1 as substrate. Reactions were stopped by addition of 30 μl of 2 times concentrated SDS sample buffer, samples were subjected to gel electrophoresis on 15% polyacrylamide gels and phosphorylated histone H1 was quantified using a Phospho-Imager (Fuji).

Flow cytometry. 10^5 cells were plated onto 6-well plates, and after 24 h incubation starved for additional 16 h and treated with inhibitors and TT-232 as indicated and incubated for 8 h in presence of 10% FCS. The monolayer of cultured cells was washed with PBS and trypsinized. Cells were centrifuged and the pellet was resuspended in −20°C ethanol. After fixation cells were washed once with 200 mM phosphate buffer (pH 7.8), resuspended in the same buffer supplemented with 100 μg/ml Rnase A and incubated for 30 min at 37°C. Analysis was performed counting 10,000 cells after propidium iodide staining in a FACScalibur flow cytometer (Beckton–Dickinson).

RESULTS

Experiments were carried out in human A431 and simian COS-7 cell lines which both express endogenous SSTR1 as shown in Fig. 1. A431 cells were chosen because they are of human origin and express only SSTR1 and no other SSTRs, COS-7 cells were used because of their high SSTR1 expression level. To confirm the assumed analogy of somatostatin and TT-232 signaling, the effect on ERK by TT-232 in A431 cells

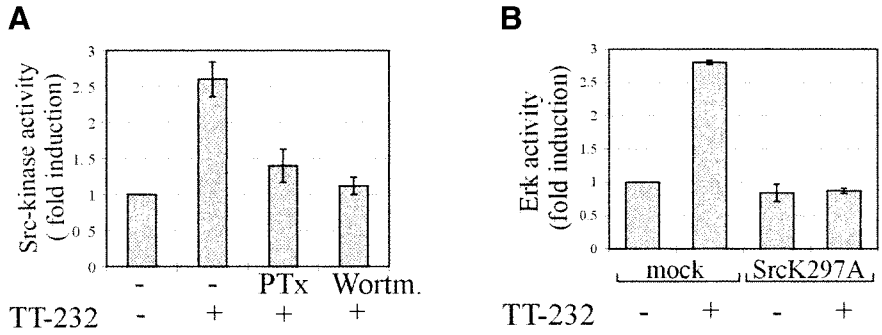

FIG. 3. Induction of c-Src activity by TT-232. (A) A431 cells were treated with 100 ng/ml pertussis toxin (PTx) for 16 h, or with 100 nM wortmannin (wortm.) for 15 min prior treatment with 50 μM TT-232 for 10 min. Cells were lysed, and c-Src activity was measured using enolase as substrate as described above. $n = 3 \pm$ SD. (B) Cos-7 cells were transfected with empty expression vector, or plasmid encoding c-Src K297A (1 μg/well) together with plasmid encoding HA-ERK2 (250 ng/well). Cells were stimulated with 50 μM TT-232 for 10 min prior to lysis and immunoprecipitated HA-ERK2 was subjected to *in vitro* kinase assay as described. $n = 4 \pm$ SD.

was tested. As shown in Fig. 2A, the activation of ERK was dependent on the time of stimulation. Activity reached a maximum after about 10 min and declined back to control levels after 30 min. This early event was prevented by treatment with the G-protein-coupled receptor inhibitor pertussis toxin or by the PI3K inhibitor wortmannin (Fig. 2B).

Florio *et al.* (14) speculated that c-Src is a mediator of somatostatin/SSTR1 signaling because overexpression of the inhibitory c-Src kinase Csk abrogated the activation of ERK upon somatostatin stimulation. Since Csk does not only inhibit c-Src but also other members of the Src family (22, 23), we chose c-Src specific assays that would allow more definite conclusions. We measured c-Src activity directly in immunoprecipitates of TT-232 treated cells. As demonstrated in Fig. 3A, a 2.5-fold induction of c-Src was found which could be inhibited by pertussis toxin and also by wortmannin. Introduction of c-SrcK297A, a kinase inactive dominant negative mutant of c-Src, completely blocked the TT-232 induced activation of ERK (Fig. 3B). This indicated that c-Src functions upstream of ERK and is necessary for triggering the ERK/MAPK pathway.

To test the hypothesis that PKCs play a role in TT-232 signaling, we used the PKC inhibitor GF109203X to block TT-232 induced activation of c-Src (Fig. 4A) and ERK (Fig. 4B). The activity of both enzymes was abolished upon pretreatment of the cells with PKC inhibitor. Therefore, we concluded that at least one PKC isoform that acts upstream of c-Src must be an essential element of the TT-232 pathway.

FACS analysis was employed to confirm that the involvement of PKC in TT-232 signaling is required for the antiproliferative effect of the somatostatin analogue. As already demonstrated in Fig. 2A, TT-232 induced ERK activation declined to basal level within 20 to 30 min of treatment of A431 cells. Therefore, starved cells were pulse treated with TT-232 for 30 min, washed and incubated in 10% FCS for further 8 h to allow them to reenter the cell cycle. As shown in Fig. 5, the antiproliferative effect of TT-232 was identified to be caused by a cell cycle arrest in G_1/S phase. After 16 h posttreatment incubation in 10% FCS the cells did not recover but slowly started to undergo apoptosis (data not shown). Thus, the effect of the pulse treatment with TT-232 can be considered as irreversible. Fifteen minutes preincubation with the PKC inhibitor GF109203X completely prevented cell cycle arrest, in agreement with the inability of TT-232 to activate c-Src and ERK after GF109203X pretreatment. Inhibition of MEK with PD98059 had the same effect, which is consistent with the concept that this kinase acts as a downstream mediator of PKC and c-Src. Furthermore, we observed that $p21^{Cip1}$ expression was induced in a time dependent manner after treatment with TT-232 (data not shown) in analogy to somatostatin (14).

Le Good *et al.* (15) showed that all members of the PKC family can bind the protein kinase B kinase PDK1. However, particularly PKCδ seemed to be activated by overexpression of PDK1, which required functional PI3K. Therefore, we asked whether PKCδ is the enzyme that mediates the GF109203X sensitive func-

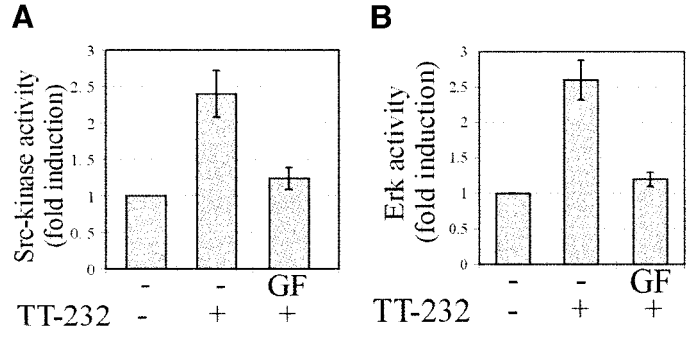

FIG. 4. PKC inhibition blocks the induction of c-Src and ERK by TT-232. Cells were pretreated with the PKC inhibitor GF109203X (0.5 μg/ml) for 15 min then treated for 10 min with 50 μM TT-232. The activity of immunoprecipitated endogenous c-Src (A) or ERK2 (B) was subjected to an *in vitro* kinase assay as described above. $n = 3 \pm$ SD.

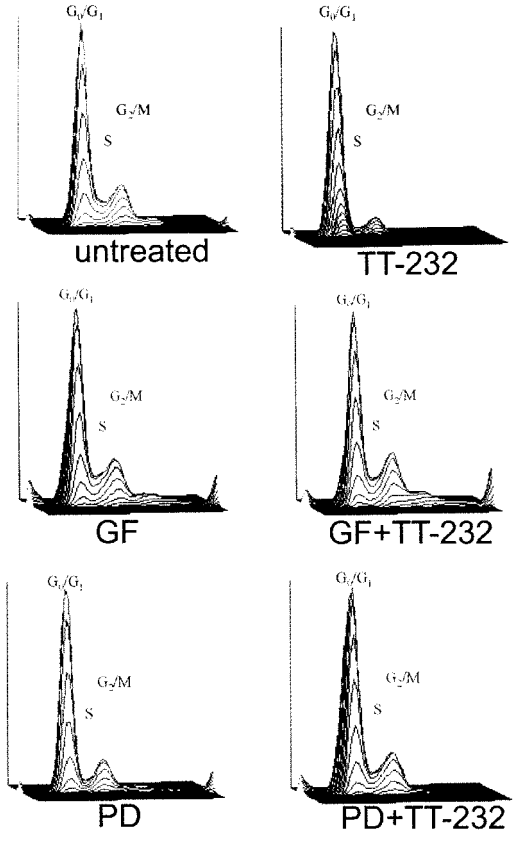

FIG. 5. Cell cycle arrest in G_1/S phase by TT-232 pulse treatment. Serum starved A431 cells were pretreated with vehicle, 25 μM PD98059 (PD), or 0.5 μg/ml GF109203X (GF) for 15 min followed by treatment with 50 μM TT-232 for 30 min. Cells were washed twice with PBS and fresh 10% FCS containing media was added for 8 h. Propidium iodine staining and FACS analysis visualized a decreased peak for the S and G_2/M population only upon TT-232 application (upper panel, right). Incubation with the inhibitors GF109203X and PD98059 prior to TT-232 abolished this effect completely (middle/lower panels).

tion of TT-232. PKCδ was immunoprecipitated from A431 cells and measured for activity upon TT-232 stimulation (Fig. 6A). The extent of induction was found to be comparable to the known PKC inducer O-tetradecanoylphorbol-13-acetate (TPA) which was included as a positive control. At the same time PKCα was found not to be affected by TT-232 (data not shown). Figure 6B shows Western blot analysis of purified cell membrane preparations. Upon TT-232 treatment, PKCδ (upper panel) but not PKCα (lowest panel) is translocated to the membrane, which generally seems to be indicative for PKC activation (24). Immunoblot analysis with an anti-EGFR antibody was used to confirm equal loading of the samples.

The PKCδ K376R mutation has already been described for its negative effect on kinase activity of PKCδ by Li *et al.* (25). Therefore, we expressed the mutant protein in COS-7 cells and investigated its impact on the phosphorylation of ERK after TT-232

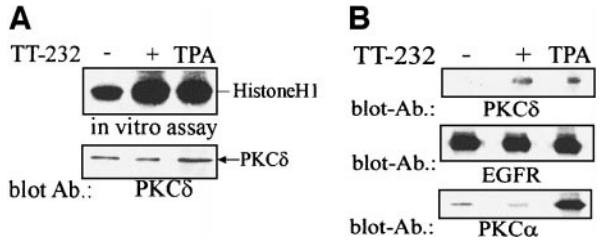

FIG. 6. Induction of PKCδ activity and translocation to the cell membrane by TT-232. (A) A431 cells were treated with 50 μM TT-232 or 10 μM TPA as positive control. PKCδ was immunoprecipitated and incubated with (γ-^{32}P) ATP and Histone H1 as substrate. (B) Cell membranes were prepared as described above and subjected to Western blot analysis as indicated. PKCδ was found in the membrane preparation after treatment with TT-232 or TPA (upper panel). PKCα was translocated by TPA but not by TT-232 (lowest panel) and anti-EGFR blot served as loading control.

treatment (Fig. 7). Mock transfection as well as expression of the wild type form of PKCδ caused no visible change in the responsiveness to TT-232. The PKCδ K376R mutant however completely abolished the phosphorylation of ERK after TT-232 treatment, which indicates that PKCδ is the only PKC family member whose function is required for TT-232 signaling in COS-7 cells.

DISCUSSION

The somatostatin analogue TT-232 has remarkable cytostatic and cytotoxic effects particularly on tumor cells. These are mediated by at least two different pathways leading to apoptosis directly or to cell cycle arrest with apoptosis as a late consequence. When cells are treated with the heptapeptide for 4 to 8 h the apoptotic pathway is induced leading to 70–100% dead cells after 24 h. Since Vántus *et al.* (26) found no change in the apoptotic effect of TT-232 after pretreat-

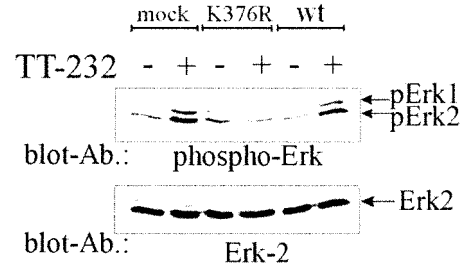

FIG. 7. PKCδ is required for the TT-232 induced phosphorylation of ERK. COS-7 cells were transfected with vector (mock), the dominant negative PKCδ mutant (K376R), or wild type PKCδ (wt). Cells were stimulated with 50 μM TT-232 for 10 min and Western blot analysis was performed using the phospho-ERK antibody (New England Biolabs, Beverly, MA), which exclusively detects phosphorylated ERK protein (upper panel). The K376R mutant but not the wild type PKCδ could prevent the phosphorylation of ERK upon TT-232 treatment. The ERK2 reblot (lower panel) warrants equal loading of the lanes.

ment of A431 cells with pertussis toxin, the G-protein coupled SSTRs do not seem to be involved in the apoptotic mechanism. This is supported by the observations that SSTR deficient CHO cells respond to TT-232 treatment with apoptosis (26) and that the PI3K inhibitor wortmannin and the MEK inhibitor PD98059 could not affect the apoptotic pathway in A431 and COS-7 cells.

Binding of TT-232 to SSTR1 leads to the transient induction of ERK activation, which returns to basal level within about 30 min. During this time, the TT-232 signal becomes irreversibly triggered. As shown by our FACS analysis, 8 h after a 30 min treatment, cells were arrested in G_1/S phase without any detectable apoptosis. These conditions allowed us to investigate the cell cycle arrest independently from the apoptosis pathway, which is only triggered when the cells are incubated with TT-232 for 4 h or longer.

We were able to demonstrate that the mechanism for the cell cycle arrest is fundamentally different from the apoptotic pathway. We related it to somatostatin signaling events mediated by SSTR1 as described by Florio *et al.* (14).

We propose a model where TT-232 binds to SSTR1 which leads to the dissociation of a receptor coupled $G_{i/o}$ protein. Subsequent release of the $G\beta\gamma$ protein complex activates PI3K (27). The PI3K product phosphatidylinositol-3,4,5-trisphosphate can induce the activity of PKCδ, which thereby translocates to the cell membrane. Furthermore, Le Good *et al.* (15) suggested the involvement of the protein kinase PDK1 in the activation of PKCs by phosphorylation. Taken together, this suggests that PI3K lipid products also recruit and activate PDK1, which in turn phosphorylates PKCδ. For the activation of PKCδ both, membrane translocation and phosphorylation are required. It is still not clear how c-Src is controlled by PKCδ, but we could clearly show that c-Src is downstream of PKCδ and that it is required for the TT-232 pathway like PKCδ itself. c-Src is a known activator of the Sos/Ras complex and the downstream MEK/ERK cascade. We provide evidence that the activation of the ERK/MAPK pathway by TT-232 inhibits the cell cycle. As it has been shown different stimuli of the MEK/ERK pathway can lead to the de novo expression of $p21^{Cip1}$, a suppressor of Cdks and thus a cell cycle inhibitor (28–30). We observed that $p21^{Cip1}$ expression was induced upon treatment with TT-232 (data not shown) in analogy to somatostatin (14). We speculate that the synthesis of $p21^{Cip1}$ requires a time interval of several hours, which would explain the delay of the cell cycle arrest after TT-232 treatment and the comparably fast subsequent signaling steps.

Since TT-232 has obviously more than one effector molecule, the signaling of the somatostatin analogue is likely to depend on the expression patterns of SSTRs and/or putative alternative binding partners, which might be located inside the cells and still have to be identified.

The somatostatin analogue TT-232 thus exhibits an ambivalent signaling capacity. Conception of such phenomena will generally improve our understanding for cellular mechanisms, their ramifications and our capacity to influence them.

ACKNOWLEDGMENT

A. Stetak was supported from 1997 to 1999 by a fellowship from the Max–Planck Institute of Biochemistry (Martinsried, Germany).

REFERENCES

1. Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., and Guillemin, R. (1973) Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179,** 77–79.
2. Reichlin, S. (1983) Somatostatin. *N. Engl. J. Med.* **309,** 1556–1563.
3. Colas, B., Cambilleu, C., Buscail, L., Zeggari, M., Estève, J. P., Lautre, V., Thomas, F., Vaysse, N., and Susini, C. (1992) Stimulation of a membrane tyrosine phosphatase activity by somatostatin analogues in rat pancreatic acinar cells. *Eur. J. Biochem.* **207,** 1017–1024.
4. Pan, M. G., Florio, T., and Stork, P. J. (1992) G-protein activation of a hormone-stimulated phosphatase in human tumor cells. *Science* **256,** 1215–1217.
5. Bauer, W., Briner, U., Doepfner, W., Halber, R., Huguenin, R., Marbach, P., Pecher, T. J., Pless, J. (1982) SMS 201–995: A very potent and selective octapeptide analogue of somatostatin with prolonged action. *Life Sci.* **31,** 1133–1140.
6. Kéri, Gy., Érchegyi, J., Horváth, A., Mező, I., Idei, M., Vántus, T., Balogh, Á., Vadász, Zs., Bökönyi, Gy., Seprődi, A., Teplán, I., Csuka, O., Tejeda, M., Gaál, D., Szegedi, Zs., Szende, B., Roze, C., Kalthoff, H., and Ullrich, A. (1996) A tumor-selective somatostatin analogue (TT-232) with strong in vitro and in vivo antitumor activity. *Proc. Natl. Acad. Sci. USA* **93,** 12513–12518.
7. Kéri, Gy., Mező, I., Horváth, A., Vadász, Zs., Balogh, Á., Idei, M., Vántus, T., Teplán, I., Mák, M., Horváth, J., Pál, K., and Csuka, O. (1993) Novel somatostatin analogues with tyrosine kinase inhibitory and antitumor activity. *Biochem. Biophys. Res. Commun.* **191,** 681–687.
8. Kéri, Gy., Mező, I., Vadász, Zs., Horváth, A., Idei, M., Vántus, T., Balogh, Á., Bökönyi, Gy., Bajor, T., Teplán, I., Tamás, J., Mák, M., Horváth, J., and Csuka, O. (1993) Structure-activity relationship studies of novel somatostatin analogues with antitumor activity. *Peptide Res.* **6,** 281–288.
9. Breder, C. D., Yamada, Y. Y., Yasuda, K., Seino, S., Saper, C. B., and Bell, G. I. (1992) Differential expression of somatostatin receptor subtypes in brain. *J. Neurosci.* **12,** 3920–3934.
10. Bruno, J. F., Xu, Y., Song, J., Berelowitz, M. (1993) Tissue distribution of somatostatin receptor subtype messenger ribonucleic acid in the rat. *Endocrinology* **133,** 2561–2567.
11. Patel, Y. C. (1999) Somatostatin and its receptor family. *Frontiers Neuroendocrin.* **20,** 157–198.
12. Patel, Y. C. (1997) Molecular pharmacology of somatostatin receptor subtypes. *J. Endocrinol. Invest.* **20,** 348–367.
13. Reisine, T., and Bell, G. I. (1995) Molecular biology of somatostatin receptors. *Endocr. Rev.* **16,** 427–442.
14. Florio, T., Yao, H., Carey, K. D., Dillon, T. J., and Stork, P. J. (1999) Somatostatin activation of mitogen-activated protein ki-

nase *via* somatostatin receptor 1 (SSTR1). *Mol. Endocrinol.* **13,** 24–37.

15. Le Good, J. A., Ziegler, H. W., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, J. P. (1998) Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281,** 2042–2045.
16. Palmer, R. H., Dekker, L. V., Woscholski, R., Le Good, J. A., Gigg, R., and Parker, P. J. (1995) Activation of PRK1 by phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate. A comparison with protein kinase C isotypes. *J. Biol. Chem.* **270,** 22412–22416.
17. Mischak, H., Goodnight, J. A., Kolch, W., Martiny-Baron, G., Schaechtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H., and Mushinski, J. F. (1993) Overexpression of protein kinase C-delta and -epsilon in NIH 3T3 cells induces opposite effects on growth, morphology, anchorage dependence, and tumorigenicity. *J. Biol. Chem.* **268,** 6090–6096.
18. Fukumoto, S., Nishizawa, Y., Hosoi, M., Koyama, H., Yamakawa, K., Ohno, S., and Morii, H. (1997) Protein kinase C delta inhibits the proliferation of vascular smooth muscle cells by suppressing G_1 cyclin expression. *J. Biol. Chem.* **272,** 13816–13822.
19. Watanabe, T., Ono, Y., Taniyama, Y., Hazama, K., Igarashi, K., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992) Cell division arrest induced by phorbol ester in CHO cells overexpressing protein kinase C-delta subspecies. *Proc. Natl. Acad. Sci. USA* **89,** 10159–10163.
20. Seedorf, K., Kostka, G., Lammers, R., Bashkin, P., Daly, R., Burgess, W. H., van der Bliek, A. M., Schlessinger, J., and Ullrich, A. (1994) Dynamin binds to SH3 domains of phospholipase C gamma and GRB-2. *J. Biol. Chem.* **269,** 16009–16014.
21. Smart, E. J., Ying, Y. S., Mineo, C., and Anderson, R. G. W. (1995) A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl. Acad. Sci. USA* **92,** 10104–10108.
22. Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M., and Aizawa, S. (1993) Constitutive activation of Src family kinases in mouse embryos that lack Csk. *Cell* **73,** 1125–1135.
23. Okada, M., Nada, S., Yamanashi, Y., Yamamoto, T., and Nakagawa, H. (1991) CSK: A protein-tyrosine kinase involved in regulation of c-Src family kinases. *J. Biol. Chem.* **266,** 24249–24252.
24. Kraft, A. S., and Anderson, W. B. (1983) Phorbol esters increase the amount of Ca2+, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* **301,** 621–623.
25. Li, W., Yu, J. C., Shin, D. Y., and Pierce, J. H. (1995) Characterization of a protein kinase C-delta (PKC-delta) ATP binding mutant. An inactive enzyme that competitively inhibits wild type PKC-delta enzymatic activity. *J. Biol. Chem.* **270,** 8311–8318.
26. Vántus, T., Krivickiene, Z., Valius, M., Steták, A., Keppens, S., Csermely, P., Bauer, P. I., Bökönyi, G., Kéri, G., Declercq, W., Vandenabeele, P., Merlevede, W., and Vandenheede, J. R. (2001) The somatostatin-analog TT-232 induces apoptosis in A431 cells: Sustained activation of stress activated kinases and inhibition of signaling to Extracellular Signal-Regulated Kinases, in press.
27. Hawes, B. E., Luttrell, L. M., Biesen, T., and Lefkowitz, R. J. (1996) Phosphatidylinositol 3-kinase is an early intermediate in the G$\beta\gamma$-mediated mitogen-activated protein kinase signaling pathway. *J. Biol. Chem.* **271,** 12133–12136.
28. Pumiglia, K. M., and Decker, S. J. (1997) Cell cycle arrest mediated by the MEK/mitogen-activated protein kinase pathway. *Proc. Natl. Acad. Sci. USA* **94,** 448–452.
29. Beier, F., Taylor, A. C., and LuValle, P. (1999) The Raf-1/MEK/ERK pathway regulates the expression of the p21(Cip1/Waf1) gene in chondrocytes. *J. Biol. Chem.* **274,** 30273–30279.
30. Hu, P. P., Shen, X., Huang, D., Liu, Y., Counter, C., and Wang, X. F. (1999) The MEK pathway is required for stimulation of p21(WAF1/CIP1) by transforming growth factor-beta. *J. Biol. Chem.* **274,** 35381–35387.