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Somatostatin inhibits PDGF-stimulated Ras activation in human neuroblastoma cells

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Abstract The main physiological role of somatostatin (SST) is the control of hormone secretion. Recently, SST has been shown to exert antiproliferative effects on some human tumors via both direct and indirect mechanisms. We have previously found that in the human neuroblastoma cell line SY5Y the SST analogue lanreotide (BIM 23014) inhibited serum-stimulated cell proliferation and MAP kinase activity. Here, we examine the effect of SST on PDGF-induced Ras activation. We found that SST suppressed PDGF-induced Ras activation in a pertussis toxin (PTx)-independent and peroxovanadate-dependent manner. Ras-specific GTPase activating protein (GAP) activities were not altered by SST treatment. On the contrary, PDGF-induced PDGF receptor phosphorylation was decreased by SST in a PTx-independent, peroxovanadate-dependent manner, likely accounting for the SST-mediated inhibition of PDGF-induced Ras activation.

Key words: Somatostatin; Human neuroblastoma; Ras activation; PDGF receptor phosphorylation

1. Introduction

Somatostatin (SST) has a broad spectrum of biological activities in different organ systems. The physiological role of hypotalamic SST on GH and TSH secretion and on the regulation of pancreatic and gastrointestinal functions is well established [1,2]. SST acts by interacting with specific membrane receptors. Five subtypes of human SST receptors (hSSTR) have been recently cloned all belonging to the family of G protein-coupled receptors with seven transmembrane spanning domains (for a review see [3]).

Recently, the use of SST or the longer lasting analogues octreotide and lanreotide to suppress cell proliferation of human tumors has been proposed [4,5]. Currently, the therapeutic application of SST analogues is limited to neuroendocrine tumors like carcinoids, GHomas, insulinomas, gastrinomas on which these drugs are thought to act by inhibiting hormone secretion. SST can inhibit secretion as well as tumor growth both indirectly, via interference with growth factor production, or directly, by interacting with specific receptors on the tumoral cells and possibly interfering with the mitogenic signalling. Finally, SST has been suggested to have antiangiogenetic properties [6].

Most of the studies on SST were performed using ectopic expression of SSTR and signal transducing molecules in cells that normally do not express them. This raises the possibility that non-physiological interactions might occur making difficult to evaluate the relevance of the results obtained in such manner. We therefore investigate the effect of SST in a cell system, the human neuroblastoma cells SY5Y, that endogenously express SSTR [7]. We have previously shown that the SST analogue lanreotide was able to inhibit cell proliferation of SY5Y cells most likely by downregulating MAP kinase activity [8]. To gain insight on the molecular mechanisms responsible for the growth suppression of SY5Y cells and MAP kinase inhibition by SST and its analogue, we investigated the possibility that SST might act on and modulate critical steps in the mitogenic signaling. The activation of the small G protein Ras is a key event for the transmission of the mitogenic signals triggered by activation of a variety of growth factor receptors including platelet-derived growth factor receptors (PDGFR). PDGFR are expressed in SY5Y cells [9]. We therefore set up to measure the effect of SST on Ras activation stimulated by PDGF by using a recently developed functional Ras assay based upon the interaction of Ras-GTP with a specific Ras binding domain (RBD) on Raf-1 [10].

2. Materials and methods

2.1. Materials

PDGF-BB was from Boehringer Mannheim, Mannheim, Germany. Peroxide of vanadate (peroxovanadate) was prepared by mixing vanadate (Sigma Chemicals, St. Louis, MO, USA) with H_2O_2 (Merck, Darmstadt, Germany) for 10 min at 37°C. All reagents were purchased from Sigma Chemicals, St. Louis, MO, USA unless otherwise indicated.

2.2. Cell culture

SY5Y cells (kindly provided by Dr. D. Fornasari, Dept. of Pharmacology, Milano, Italy) were routinely grown in RPMI-1640 medium (Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). Mouse fibroblasts (kindly donated by Dr. T. Pawson, Dept. of Molecular and Medical Genetics, Toronto, Ont., Canada) were grown in DMEM medium (Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% FBS. All cell lines were kept at 37°C in a humidified atmosphere of 5% CO_2 in air.

2.3. Ras activity assay

Gst-RBD (aa 51–131 of Raf-1) fusion protein was isolated in our laboratory from pGEX 2T-RBD induced with IPTG as previously described [10]. Activated Ras-GTP was precipitated from whole cell lysates (prepared in lysis buffer containing 50 mM HEPES, pH 7.4; 250 mM NaCl; 5 mM $MgCl_2$; 10% glycerol; 1% Triton X-100; 0.5 mM phenylmethylsulfonyl fluoride (PMSF); 10 μg/ml leupeptin and 10 μg/ml aprotinin) with 20 μl glutation agarose beads coupled with Gst fusion protein containing the Ras binding domain (RBD) of Raf. After 12% SDS-PAGE, active Ras protein was detected by West-

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Abbreviations: SST, somatostatin; PDGF, platelet-derived growth factor; MAP kinase, mitogen-activated protein kinase; PTx, pertussis toxin; GAP, GTPase activating protein

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ern blotting using monoclonal anti-Ras antibodies (Transduction Laboratories, Lexington, KY, USA).

2.4. GAP assay on cell lysates

Cell lysates were prepared using a lysis buffer containing 25 mM Tris-HCl pH 7.5; 100 mM NaCl; 1% NP-40; 1% glycerol; 10 mM $MgCl_2$; 1 mM dithiothreitol (DTT); 1 mM Na_3VO_4; 0.5 mM PMSF; 10 μg/ml leupeptin and 10 μg/ml aprotinin. The purified Ras protein (500 ng), kindly provided by Dr. M. Vanoni (Dept. of General Physiology and Biochemistry, Dept. of Biology, Milano, Italy), was loaded with 1 μM [γ-^{32}P]GTP as described [11]. The Ras-GTP complex (50 ng) was incubated with cell lysates (100 μg) or lysis buffer at room temperature in GAP buffer (25 mM Tris-HCl pH 7.5; 100 mM NaCl; 10 mM $MgCl_2$; 1 mg/ml BSA; 2 mM GTP; 0.1 mM DTT). At intervals, aliquots were removed and the GAP activity was determined by measuring the amount of protein-bound radioactive GTP remaining on the filter, by liquid scintillation counting. The results are expressed as the amount of cpm of non-hydrolyzed GTP remaining on the filters. Background counts were less than 2% of total bound [γ-^{32}P]GTP. Each time point was measured in triplicate and the values are expressed as the mean ± S.E.M.

2.5. MAP kinase activity assay

Quiescent SY5Y cells, plated in 60 mm diameter Petri dishes, were washed with phosphate-buffered saline (PBS) and lysed in 600 μl of lysis buffer containing 50 mM Tris-HCl, pH 7.5; 100 mM NaCl; 5 mM EDTA; 1 mM DTT; 40 mM $Na_4P_2O_7$; 0.2 mM Na_3VO_4; 1% Triton X-100; 1 mM PMSF; 10 μg/ml aprotinin; 10 μg/ml leupeptin and 10 μg/ml pepstatin. After a 15 min centrifugation at 13 000 × *g* at 4°C, MAP kinase was immunoprecipitated from the lysates using a polyclonal anti-ERK 2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein A-Sepharose beads. Immunocomplex kinase assay was performed for 30 min at 30°C in kinase buffer (containing 40 mM HEPES-NaOH, pH 7.5; 5 mM magnesium acetate; 2 mM DTT; 1 mM EGTA; 0.2 mM Na_3VO_4; 1 mM PMSF; 10 μg/ml aprotinin; 10 μg/ml leupeptin and 10 μg/ml pepstatin) using myelin basic protein (250 μg/ml, MBP, Gibco BRL, Grand Island, NY, USA) and [γ-^{32}P]ATP (3.0 μCi/sample; specific activity 3000 Ci/mmol; Amersham, UK) as substrates. An aliquot of the supernatants was transferred onto P81 phosphocellulose paper squares and after five washes in 0.75% phosphoric acid the radioactivity associated to the squares was determined by liquid scintillation counting.

2.6. Immunoprecipitation and immunoblotting

Quiescent SY5Y cells, plated in 100 mm diameter Petri dishes, were washed with PBS and lysed in 500 μl of lysis buffer containing 50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM EGTA; 1% NP-40; 0.25% sodium deoxycholate; 1 mM Na_3VO_4; 1 mM NaF; 1 mM PMSF; 10 μg/ml aprotinin; 10 μg/ml leupeptin and 10 μg/ml pepstatin. After a 20 min incubation at 4°C, the lysate was collected and centrifuged at 13 000 × *g* for 10 min at 4°C. Proteins of interest were immunoprecipitated using a polyclonal anti-PDGF receptor antibody (Upstate Biotechnology, Lake Placid, NY, USA) or a monoclonal anti-phosphotyrosine antibody (PY20, Transduction Laboratories, Lexington, KY, USA). The immunocomplexes were separated by 7.5% SDS-PAGE, transferred on nitrocellulose membranes and the filters incubated with the appropriate antibodies. The immunoreactive bands were then detected by ECL system (Amersham, UK).

2.7. Densitometric analysis

The densitometric analysis of the immunoblots was performed using the NIH Image 1.44 program. Each blot was analyzed by a single operator that was unaware of treatment groups. Data are expressed as mean ± S.D. of the percentage of inhibition measured in SST plus PDGF-treated samples versus PDGF-treated samples, considered as 100%.

3. Results

Serum-deprived SY5Y cells were stimulated with 20 ng/ml of PDGF with or without pretreatment with SST (10 nM). Total cellular lysates were immunoprecipitated with Gst-RBD (the minimal Ras binding domain of Raf) which preferentially binds to the GTP-bound active form of Ras. As shown in Fig. 1A, PDGF stimulation of cells (lane 2) increased the amount of active Ras bound to Gst-RBD relative to the amount of activated Ras present in lysates of untreated cells (lane 1). Pretreatment of SY5Y cells with SST abrogated almost completely the formation of Ras-GTP induced by PDGF (lane 3). Densitometric analysis of the immunoblots showed an inhibition of 61.1 ± 18.4% ($n = 6$) caused by SST which had no effect on unstimulated Ras activity (lane 4). Equal amount of total Ras proteins were subjected to the RBD binding assay as shown by immunoblotting of an aliquot of the same lysates used for the assay followed by detection with anti-Ras antibodies (Fig. 1A, lower panel). The reduction in the level of activated Ras was paralleled by an inhibition of 44.0 ± 2.96% ($n = 3$), of PDGF-induced MAP kinase activity as measured by immunocomplex kinase assay (Fig. 1B), in agreement with our previous data [8].

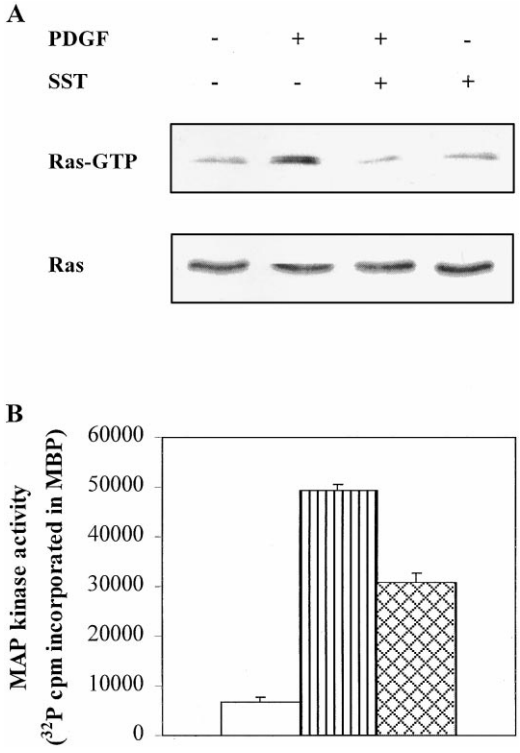

Fig. 1. Effect of SST on PDGF-induced Ras and MAP kinase activation. A: Serum-deprived SY5Y cells were pretreated for 8 min with SST (10 nM) and then stimulated with PDGF-BB (20 ng/ml) for 4 min before cell lysis. Ras was identified either by affinity precipitation with Gst-RBD followed by immunoblotting (upper panel) or by immunoblotting of total cell lysates (lower panel) using anti-Ras antibodies. This experiment was repeated six times with similar results. B: Serum-deprived SY5Y cells were treated as above with medium alone (open bar), PDGF (vertical bar) or SST and PDGF (cross-hatched bar). MAP kinase activity was measured by immunocomplex kinase assay as described in Section 2. Data are expressed as cpm of ^{32}P incorporated in MBP and are the mean ± S.E.M. of three independent experiments, each performed in duplicate.

The biological effects of SST are mediated through a family of G protein-coupled receptors of which five members have been identified by molecular cloning [3]. All five hSSTRs are functionally coupled to inhibition of adenylate cyclase via PTx-sensitive GTP binding proteins [12]. In SY5Y cells pretreated for 4 h with PTx (100 ng/ml), SST was still capable of inhibiting PDGF-induced Ras activation (Fig. 2A). The effi-

cacy of the toxin in these cells is demonstrated by the shift of the ADP ribosylated $G\alpha_{i1}$ and α_{i2} subunits caused by the 4 h treatment with PTx (Fig. 2A, insert). These data indicate that the coupling of the SST receptor to the effector responsible for the inhibitory action on Ras does not occur via a PTx-sensitive G protein raising the possibility that SST might act through coupling of SSTR to a PTx-resistant G protein. Indeed, a PTx-resistant $G\alpha_{12}$ subunit was shown to be capable of reducing the amount of Ras-GTP stimulated by EGF in COS-7 cells by activating the Ras GPTase activating protein $Gap1^m$ [13]. This finding prompted us to assess whether SST-dependent inhibition of Ras activation could be due to the ability of SST to modulate Ras-specific GAP activities. To measure GAP activities, a recombinantly produced and purified Ras protein was loaded with [γ-^{32}P]GTP and incubated with total cellular lysates obtained from SY5Y cells. As shown in Fig. 3A, the addition of cellular lysates increased the rate of GTP hydrolysis as compared to the addition of lysis buffer alone as a control. However, no differences in GAP activity

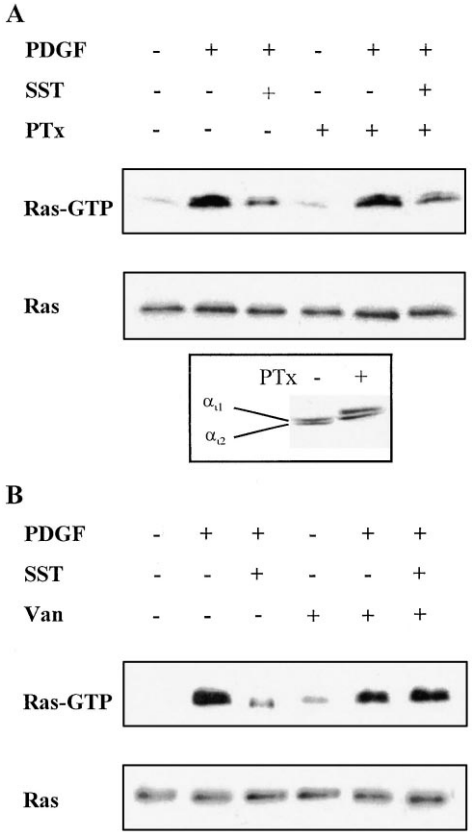

Fig. 2. Effect of Pertussis toxin and sodium peroxovanadate treatment on SST-induced inhibition of Ras activity stimulated by PDGF. A: Where indicated, serum-deprived SY5Y cells were preincubated for 4 h with PTx (100 ng/ml) prior to assay. Cells pretreated with SST (10 nM) for 8 min were then stimulated with PDGF-BB (20 ng/ml) for 4 min. Ras was identified in cell lysates as described in Fig. 1A. This experiment was repeated 12 times with similar results. In the insert, the shift of the ADP-ribosylated α_{i1} and α_{i2} G protein subunits following PTx pretreatment is presented. B: Serum-deprived SY5Y cells were pretreated with sodium peroxovanadate (25 μM) for 10 min where indicated. Prior to stimulation with PDGF-BB (20 ng/ml) for 4 min, the cells were treated with SST (10 nM) for 8 min. Ras was identified in cell lysates as described in Fig. 1A. This experiment was repeated five times with similar results.

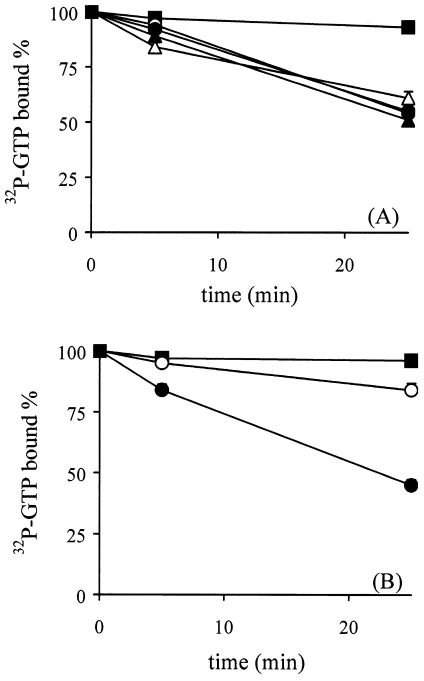

Fig. 3. Effect of SST on Ras-specific GAP activities in SY5Y cells and Ras-Gap1 +/+ and −/− mouse fibroblasts. A: Purified Ras protein was loaded with [γ-^{32}P]GTP. The Ras-GTP complex was incubated with 100 μg of total cellular lysates obtained from serum-deprived SY5Y cells that were either untreated (○) or treated with SST (10 nM) (△) or PDGF-BB (20 ng/ml) (●), or SST and PDGF (▲). Controls were performed with lysis buffer alone (■). B: GAP activity was measured by incubating the Ras-GTP complex with 100 μg of total cellular lysates obtained from wild-type (●) or Ras-GAP −/− (○) fibroblasts. Controls were performed as in A (■). At the indicated time points, aliquots were subjected to filter binding assays, as described in Section 2. The GAP activity is expressed as total cpm of non-hydrolyzed [γ-^{32}P]GTP which remained bound on the filters. Values are the mean ± S.E.M. of three independent experiments each performed in triplicate.

could be observed in lysates obtained from SY5Y cells whether or not PDGF or SST or a combination of the two were used to treat the cells. This was not due to the inability of the assay to detect differences in GAP activities as demonstrated by GAP assays performed on total cellular lysates derived from fibroblasts in which the Ras-GAP gene was genetically removed [14]. As shown in Fig. 3B, the rate of hydrolysis of GTP-loaded Ras of lysates from Ras-GAP −/− cells was substantially decreased when compared to the one measured in lysates from wild-type fibroblasts. Thus, activation of a Ras-specific GAP is unlikely to be responsible for the observed SST-dependent, PTx-independent inhibition of Ras activation.

Individual subtypes of SSTR have been shown to be linked to multiple transmembrane effectors other than adenylate cyclase. For example, SST has been shown to stimulate a tyrosine phosphatase activity [15–18] which appears to be responsible for SST-mediated antiproliferative effect [16–18]. To test whether a tyrosine phosphatase activity is involved in the inhibitory effect of SST on Ras activation, we pretreated the cells with peroxovanadate, an inhibitor of tyrosine phosphatase activity (for a review see [19]). As shown in Fig. 2B, peroxovanadate reverted the inhibitory effect of SST on PDGF-induced Ras activity, suggesting that the activity of a protein tyrosine phosphatase is involved in the SST effect.

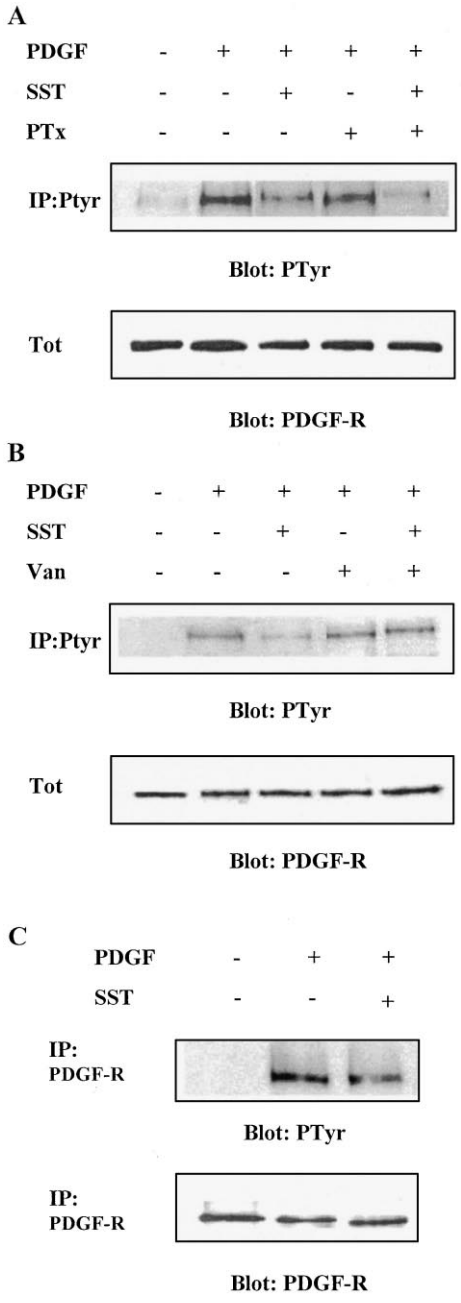

Fig. 4. Effect of SST on the PDGF receptor phosphorylation. Lysates from SY5Y cells stimulated for 2 min with PDGF-BB (20 ng/ml) with or without pretreatment with SST (10 nM) were subjected to immunoprecipitation with anti-phosphotyrosine (A, B) or anti-PDGF receptor (C) antibodies. Immunoprecipitates were resolved by 7.5% SDS-PAGE and analyzed by immunoblotting with the indicated antibody. A Western blot of the PDGFR in an aliquot of the total cell lysates (A and B) or of the immunoprecipitates (C) is presented under each experiment. In A and B cells were also pretreated with PTx (100 ng/ml for 4 h) or peroxovanadate (25 μM for 10 min) respectively before exposure to SST and PDGF. Cell lysates were subjected to immunoprecipitation with anti-phosphotyrosine antibodies and the immunoprecipitates were analyzed as described above. Each type of experiment was repeated five times with similar results.

Peroxovanadate per se slightly increased active Ras (lane 4). We then analyzed the level of protein phosphorylation induced by PDGF in presence of SST pretreatment. Quiescent SY5Y cells were treated with PDGF for 2 min with or without SST pretreatment. Total cellular lysates were subjected to immunoprecipitation either with anti-phosphotyrosine (P-Tyr) or anti-PDGF receptor (PDGF-R) antibodies (Fig. 4A,B and C). The immunoprecipitates were then resolved by 7.5% SDS-PAGE and examined by immunoblotting with anti-P-Tyr antibody. As shown in Fig. 4A and B, a prominent band of apparent molecular weight of 180 kDa appeared tyrosine phosphorylated upon PDGF stimulation. Pretreatment with SST decreased the phosphorylation of the 180 kDa band by $40.0 \pm 17.2\%$ ($n = 11$) as determined by densitometric analysis (Fig. 4A and B, three first lanes). To assess whether the band of 180 kDa corresponds to the PDGF receptor itself, we immunoprecipitated PDGF receptor with anti-PDGFR-specific antibodies and looked at its phosphorylation levels upon pretreatment with SST followed by PDGF stimulation. As shown in Fig. 4C, the phosphorylation of PDGFR induced by PDGF was decreased by $48.0 \pm 5.9\%$ ($n = 5$) following pretreatment with SST. Western blotting with anti-PDGFR antibody of an aliquot of the total cellular lysates (Fig. 4A and B, lower panel) and of the immunoprecipitates (Fig. 4C, lower panel) showed that equal amounts of proteins were present in all the samples. We then examined the phosphorylation levels of PDGF receptor with or without SST pretreatment in cells that were incubated for 4 h with PTx (100 ng/ml). As shown in Fig. 4A, SST inhibited PDGF-induced PDGF receptor phosphorylation also in the presence of Ptx. Conversely, vanadate pretreatment reversed the inhibitory effect of SST on PDGF receptor phosphorylation (Fig. 4B). Thus, the inhibitory effect of SST on PDGF receptor phosphorylation is PTx-insensitive and vanadate-sensitive similarly to the hormone inhibitory effect on Ras.

4. Discussion

The present study was undertaken to gain insight into the molecular mechanism of SST inhibition of growth factor-dependent cell proliferation in SY5Y neuroblastoma cells. Ras plays a key role in the transduction of mitogenic signals by external stimuli, including polypeptide growth factors like PDGF. Here, we show that SST inhibits PDGF-induced Ras activation in human neuroblastoma cells. Gi-coupled receptors have been shown to activate Ras via a mechanism that involves the βγ subunits of the Gi protein [20]. Our results show for the first time that a Gi-coupled receptor may instead inhibit Ras activity. The inhibition by SST on Ras is not sensitive to PTx raising the possibility that SST acts by coupling SSTR to PTx-insensitive Gi protein. All five hSST receptors cloned are coupled to a variety of intracellular signals including the inhibition of adenylate cyclase and L-type calcium channel and the activation of K^+ channels all of which are mediated by PTx-sensitive G proteins. Recently, however, the PTx-insensitive G protein subunit α_{12} has been shown to directly bind and activate one of the Ras-GTPase activating proteins, $GAP1^m$, and thus negatively regulates Ras signalling in COS cells [13]. A similar mechanism, however, does not seem to account for the observed SST-dependent inhibition of Ras activation since SST has no effect on Ras-specific GAP activities measured in total cellular lysates of SY5Y cells.

An alternative possible mechanism of action given the PTx-insensitivity of the SST effect, is the direct association of SSTR to a downstream effector molecule by-passing the requirement for coupling of SSTR to G proteins. Recently, the

Na^+/H^+ exchanger was shown to be regulated by the β_2 adrenergic receptor via an intermediate protein bearing a PDZ domain which binds to a consensus sequence on the C-terminal tail of the receptor [21]. The same consensus sequence (T/SXV/L/I) is present on the C-terminal tail of all the SSTR subtypes with the exception of the SSTR4. This raises the possibility that SST inhibition of Ras activation might be mediated by the association of the receptor with a protein bearing a PDZ domain acting either directly as an effector or indirectly as an adapter molecule. The identification of such a molecule would allow to test its involvement in SST inhibition of PDGF-induced Ras activation and efforts in this direction are actually ongoing in our laboratory.

Peroxovanadate treatment of cells abrogates the inhibitory effect of SST on PDGF-stimulated Ras activity suggesting that a tyrosine phosphatase might be involved in mediating SST action. That a tyrosine phosphatase could be the SST effector is further supported by the inhibition of the PDGFR tyrosine phosphorylation caused by SST. The reduction in the receptor phosphorylation level may hamper or reduce the downstream signaling leading to Ras activation. To identify such phosphatase and the nature of its coupling to the SST receptor will be instrumental in defining novel molecular mechanisms underlying not only the SST action, but also the cross-talk between receptor tyrosine kinases and heptahelical receptor-mediated signaling networks.

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