Co-purification of a protein tyrosine phosphatase with activated somatostatin receptors from rat pancreatic acinar membranes

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We have previously shown that somatostatin promotes the stimulation of a membrane tyrosine phosphatase activity in pancreatic cells. To gain insight into the mechanism of somatostatin action, we purified somatostatin-receptor complexes from somatostatin 28-prelabelled rat pancreatic plasma membranes by immunoaffinity chromatography using immobilized antibodies raised against the N-terminal part of somatostatin 28, somatostatin 28 (1–14), which is not involved in receptor-binding-site recognition. After SDS gel electrophoresis a band with a molecular mass of 87 kDa was identified in the affinity-purified material as the somatostatin receptor. The 87 kDa protein was not observed when the membrane receptors were solubilized in a free unoccupied or somatostatin 14-occupied form, or when non-immune serum replaced the anti-[somatostatin 28 (1–14)] antiserum. Somatostatin 14 inhibited the appearance of the 87 kDa protein in the same range of concentrations that inhibit radioligand binding on pancreatic membranes. After somatostatin 28 treatment of membranes, purified somatostatin receptor preparations exhibited an elevated tyrosine phosphatase activity that dephosphorylated phosphorylated epidermal growth factor receptor and poly(Glu,Tyr). This activity was related to the presence of somatostatin receptors in purified material. It was increased by dithiothreitol and inhibited by orthovanadate. In purified material containing somatostatin receptors, anti-[Src homology 2 domains (SH2)]-containing tyrosine phosphatase SHPTP1 polyclonal antibodies identified a protein of 66 kDa which was not detected in the absence of somatostatin receptor. Furthermore, the anti-SHPTP1 antibodies immunoprecipitated specific somatostatin receptors from somatostatin-prelabelled pancreatic membranes and from untreated membranes. These results indicate that a 66 kDa tyrosine phosphatase related to SHPTP1 co-purifies with the pancreatic somatostatin receptors, and suggest that this protein is associated with somatostatin receptors at the membrane level.

INTRODUCTION

Somatostatin, a tetradecapeptide originally isolated from the hypothalamus, is a widely distributed peptide exerting inhibitory effects on various cellular functions including secretory processes [1–3], neurotransmission and cell proliferation [4,5]. Somatostatin induces its biological effects by interacting with membrane-bound specific high-affinity receptors which have been characterized in the brain and peripheral tissues [6,7]. Pharmacological studies involving different radiolabelled somatostatin analogues have suggested the existence of multiple subtypes of somatostatin receptors [6–8]. Indeed, attempts to identify somatostatin receptors using photoaffinity-labelling techniques have yielded molecular masses ranging from 58 to 90 kDa for different target cells. A putative membrane somatostatin receptor of molecular mass 60 kDa has been purified from rat brain [9] whereas a protein of molecular mass 85–90 kDa has been purified from GH_4C_1 pituitary and gastric tumoural cell line [10,11]. The existence of heterogeneities in the structure of the somatostatin receptors is emphasized by the recent cloning of five different somatostatin receptors, SSTR1–SSTR5, which have distinct tissue distributions [12–15]. These receptors are members of the superfamily of G-protein-coupled receptors with seven α-helical transmembrane segments.

In most of the cells, somatostatin receptors are negatively coupled to adenylate cyclase via a pertussis toxin-sensitive inhibitory G_i protein [4,16]. Somatostatin receptors interact with other cellular effector systems such as K^+ and Ca^{2+} channels through pertussis toxin-sensitive G proteins [17–19]. Finally, somatostatin can also induce protein dephosphorylation [20,21].

In normal and tumoural pancreatic acini, we recently characterized a membrane tyrosine phosphatase activity and demonstrated that somatostatin stimulation of cells increases the enzyme activity at concentrations involving receptor occupancy [22,23]. To address further the mechanisms involved, we have tested the hypothesis that somatostatin-stimulated tyrosine phosphatase is specifically associated with somatostatin receptor and occurs in the somatostatin-receptor-enriched membrane fraction of pancreatic cells. In this study, we report a new procedure to purify activated pancreatic somatostatin receptors by immunoaffinity chromatography which allows us to demonstrate that a phosphotyrosine phosphatase co-purifies with somatostatin receptors.

EXPERIMENTAL

Materials

Somatostatin 28 (S28) and somatostatin 14 (S14) were gifts from Dr. L. Moroder (Max-Planck Institute, München, Germany), somatostatin analogues, SMS 201–995 (SMS) and [Tyr^3]SMS from Dr. Marbach (Sandoz Pharma, Basle, Switzerland) and the tyrosine analogue [Tyr^{15}]S28 from Dr. J. Rivier (Salt Lake Institute, San Diego, CA, U.S.A.). The peptide S28 (1–14) was obtained by solid-phase synthesis. Alkaline phosphatase conjugate substrate kit was purchased from Bio-Rad. Protein A–Trisacryl GF-2000 was from IBF. Purified recombinant glutathione S-transferase fusion protein, containing full-length

Abbreviations used: S28, somatostatin 28; S14, somatostatin 14; SMS, SMS 201–995; EGF, epidermal growth factor; SH2, Src homology 2 domain.

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human tyrosine phosphatase SHPTP1, was from Upstate Biotechnology, Inc., Lake Placid, U.S.A.

Plasma membrane preparation and solubilization of S28-prelabelled membranes

Pancreatic acinar cell plasma membranes were prepared as described previously [24] with ovalbumin instead of BSA. Binding of S28 was carried out by incubating membranes (100 μg/ml) for 75 min at 25 °C in 50 mM Tris/HCl buffer, pH 8.0, containing 0.2 mM $CaCl_2$, 0.5 mM bacitracin, 0.1 mg/ml soybean trypsin inhibitor and 1 mg/ml ovalbumin with 1 nM S28 in the presence or absence of 1 μM S14 as previously described [24,25]. In these conditions, displaceable binding represented about 50% of total binding capacity and non-specific binding was less than 10% of total binding as determined by Scatchard analysis using iodinated somatostatin analogues [26]. After centrifugation, the membranes were solubilized by adding to the pellet (5 mg/ml) buffer A [50 mM Tris/HCl buffer (pH 8) containing 5% (w/v) glycerol, 0.2 mM $CaCl_2$] and 1% CHAPS. The mixture was gently agitated for 1 h at 4 °C and thereafter centrifuged at 400000 *g* for 10 min in a Beckman model TL100 ultracentrifuge. The supernatant was loaded on to a Sephadex G-25-SF column (11 cm × 0.8 cm) at 4 °C equilibrated in buffer B [20 mM Tris/HCl buffer (pH 7.8) containing 0.2% CHAPS, 100 mM NaCl, 5% glycerol] and 1 mg/ml ovalbumin. Solubilized somatostatin–receptor complexes were eluted in the void volume of the column as described [25] and supplemented with 0.1 mg/ml soybean trypsin inhibitor.

Immunoaffinity chromatography

The anti[S28 (1–14)] serum was produced as described previously [27] and affinity purified by Protein A–Sepharose chromatography. A sample (1 ml) of purified IgG fraction (16 mg) was immobilized by cross-linking to 1 ml of Protein A–Trisacryl GF-2000 matrix using dimethyl pimelimidate according to [28]. About 7 mg of IgG were coupled per ml of gel (as estimated by absorbance at 280 nm of the supernatant). The immobilized antibody was subsequently washed and stored at 4 °C in 20 mM Tris buffer, pH 7.4, containing 0.02% CHAPS and 0.02% sodium azide.

Preliminary experiments using an iodinated analogue of S28, ^{125}I-[Tyr^{15}]S28, allowed us to determine optimal conditions for immune recognition and elution of S28–receptor complexes. ^{125}I-[Tyr^{15}]S28 bound to purified pancreatic membranes according to a saturable process similar to that we previously reported for other iodinated somatostatin analogues [25,26] with an apparent K_D of 180 pM and a $B_{max.}$ of 2.9 pmol/mg of protein (results not shown). After solubilization of ^{125}I-[Tyr^{15}]S28-prelabelled membranes with 1% CHAPS and gel filtration, soluble ^{125}I-[Tyr^{15}]S28–receptor complexes were then incubated for different times with immobilized anti-[S28 (1–14)] antibody in the presence or absence of S28 (1–14). Specific binding of radiolabelled material to antiserum reached a maximum after a 40 h incubation at 4 °C and remained stable for at least 4 days. In these conditions, 45–50% of the applied radioactivity were specifically recognized by the antiserum (results not shown). The immunomatrix was then poured into a mini column and washed twice with 100 vol. of buffer B containing 0.5 mg/ml ovalbumin and 1 mg/ml soybean trypsin inhibitor and four times with 100 vol. of buffer A containing 0.2% CHAPS and 50 mM NaCl (elution buffer). No specific binding was detected in these washing fractions. ^{125}I-[Tyr^{15}]S28–receptor complexes were eluted from the immunoaffinity matrix by addition of 1×10^{-4} M S28 (1–14) in elution buffer for two 24 h incubation periods, which allowed specific elution of about 50% of the bound radioactivity.

Thus immunoaffinity purification of the somatostatin receptor was routinely conducted on solubilized S28-prebound membranes (7 mg of proteins), incubated with immobilized antibody (30 μl) for 40 h at 4 °C and the bound material was washed as described above and was eluted twice with 1 ml of the elution buffer supplemented with 1×10^{-4} M S28 (1–14) peptide. Eluted proteins were concentrated in Centricon 30 tubes (Amicon), supplemented with sample buffer (50 mM Tris, pH 6.8, 5% SDS, 5% glycerol and 0.01% Bromophenol Blue) containing 50 mM dithiothreitol and heated for 15 min at 60 °C for SDS/PAGE analysis [29] and staining [30]. For dephosphorylation assays and Western blotting experiments, the material bound to immunomatrix was washed once with 100 vol. of elution buffer and was eluted twice with S28 (1–14) peptide as previously described. Then washing and eluted fractions were concentrated in Centricon 10 tubes.

Immunoblotting and immunoprecipitation

To generate polyclonal rabbit anti-SHPTP1 antibodies, a peptide corresponding to a common carboxyl region peptide (DCDIDIQKTIQMVRA) of rat SHPTP1 (C. Cambillau, unpublished work) and human SHPTP1 [31] was synthesized (a gift from Dr. P. Robberecht and Dr. A. Vandermers, Department of Biochemistry, Free University of Brussels, Brussels, Belgium), conjugated to keyhole limpet haemocyanin and injected into male rabbits. The anti-SHPTP1 antibodies were then affinity-purified by Protein A–Sepharose chromatography and used at a 1:1000 dilution for immunoblotting. Samples of eluted immunopurified receptor complexes and tyrosine phosphatase SHPTP1 were electrophoresed as described by Laemmli [29] on 10% (w/v) polyacrylamide gel and the proteins were electrotransferred to nitrocellulose membrane according to Svoboda et al. [32]. The nitrocellulose membranes were saturated with 2% (w/v) ovalbumin solution in 20 mM Tris/HCl buffer, pH 7.6, containing 137 mM NaCl and 0.05% Tween 20, and then incubated for 4 h at room temperature with anti-SHPTP1 antibodies. After washing three times with TBST, the detection of bound antisera was performed with alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibody. Immunoblots were developed in a solution of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate tolidium salt.

For immunoprecipitation, pancreatic acinar membranes were prepared as described [22], labelled with 1 nM ^{125}I-[Tyr^{3}]SMS for 90 min at 25 °C in the presence or absence of 1 μM SMS, and ^{125}I-[Tyr^{3}]SMS–receptor complexes were solubilized as described above. Soluble ^{125}I-[Tyr^{3}]SMS–receptor complexes were then incubated for 16 h at 4 °C with anti-SHPTP1 antibodies at a 1:10 final dilution. Protein A–Sepharose beads were prewashed three times, added to the samples and incubated 3 h at 4 °C. The samples were centrifuged at 1000 *g* for 5 min. The pellets were washed three times with 50 mM Tris/HCl buffer/0.1 mg/ml soybean trypsin inhibitor/1 mg/ml BSA and the radioactivity was measured.

Immunoprecipitation was also carried out from non-prelabelled soluble pancreatic somatostatin receptors as described above. Somatostatin receptors were then detected in the immunoprecipitates as described [24]. Briefly, the immunoprecipitates were incubated with 0.2 nM ^{125}I-[Tyr^{3}]SMS in buffer A containing 0.1 mg/ml soybean trypsin inhibitor and 1% (w/v) BSA. Non-specific binding was determined in the presence of 1 μM SMS. The binding reaction was carried out for 90 min at 25 °C. Prewashed Protein A–Sepharose beads were added to the samples

and incubated for 3 h at 4 °C. The samples were centrifuged at 1000 *g* for 5 min and the radioactivity was measured in the pellet. The supernatant was removed and the same volume of a solution containing 0.4 mg/ml bovine γ globulins, 2 mg/ml KI and 10% poly(ethylene glycol) was added. Samples were vacuum-filtered over Whatman (GF/C) glass-fibre filters which were presoaked for 2 h in 0.5% polyethylenimine at 4 °C. The filters were washed twice with 5 ml of ice-cold 10% poly(ethylene glycol) solution and the radioactivity was measured.

Dephosphorylation of ^{32}P-labelled epidermal growth factor (EGF) receptor from A431 cell membranes

EGF receptor from A431 cell membranes (12 μg of protein/20 μl) was autophosphorylated in a reaction mixture containing 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM Na_3VO_4, 300 nM EGF (phosphorylation buffer). After preincubation for 10 min at 20 °C, the receptor phosphorylation was initiated by addition of 8 μCi of [γ-^{32}P]ATP (3000 Ci/mmol). The reaction was continued for 10 min at 20 °C and terminated by the addition of 10 vol. of phosphorylation buffer containing 20 mM EDTA. The membranes were sedimented for 10 min at 10000 *g* and the pellet was used for tyrosine phosphatase assay. A standard reaction contained 10 μl of autophosphorylated A431 membranes (60000 c.p.m.) in 50 mM Tris/HCl, pH 7, containing 0.2 mM $CaCl_2$, 0.3 mg/ml soybean trypsin inhibitor, 0.5 mg/ml bacitracin, 0.1 mM phenylmethanesulphonyl fluoride, 5% glycerol, 5 mM dithiothreitol and 0.1% ovalbumin (dephosphorylation buffer). The reaction was initiated by the addition of immunoaffinity-purified proteins. After a 10 min incubation at 30 °C, the membranes were sedimented and the pellet was incubated with sample buffer for 15 min at 60 °C. Samples were analysed by SDS/PAGE and labelled proteins were revealed by autoradiography. Dephosphorylation of labelled proteins was quantified by image analysis using a Biocom apparatus (Biocom, Paris, France).

Assay for phosphotyrosine phosphatase activity

Poly(Glu,Tyr) was phosphorylated as previously described [22]. Tyrosine phosphatase activity was measured by the release of [^{32}P]P_i from phosphorylated poly(Glu,Tyr) in a 150 μl reaction mixture containing 30000 c.p.m. of ^{32}P-poly(Glu,Tyr) and immunoaffinity-purified proteins in dephosphorylation buffer. For measurements of tyrosine phosphatase activity of purified SHPTP1, the dephosphorylation reaction was performed under the same conditions with different concentrations of the enzyme (5–250 ng/assay). The reaction was allowed to proceed for 10 min at 30 °C then stopped by addition of 150 μl of 30% trichloroacetic acid. The [^{32}P]P_i liberated was extracted using the molybdate extraction procedure and radioactivity was evaluated by liquid-scintillation counting [22,23]. The amount of [^{32}P]P_i released was determined from the specific radioactivity of [γ-^{32}P]ATP used for the phosphorylation reaction. One unit of phosphotyrosine phosphatase activity was defined as the amount which released 1 nmol of phosphate/min at 30 °C from labelled substrate.

Protein determination

Protein concentrations of membrane and detergent-solubilized preparations were determined by the Bradford procedure using BSA as standard [33]. Proteins in the fractions of immunoaffinity chromatography were quantified after SDS/PAGE by image analysis using a Biocom apparatus with molecular-mass markers (Bio-Rad) as standards.

RESULTS

Purification of somatostatin–receptor complexes by immunoaffinity chromatography

Purification of somatostatin receptors was based on the property of anti-[S28 (1–14)] antibodies to specifically immunoprecipitate solubilized S28–receptor complexes but not S14–receptor complexes. Indeed, the antibody cross-reacted with the N-terminal fragment of S28 but not with the C-terminal part of the molecule which is common to S28 and S14 and is involved in the recognition of the somatostatin-binding site [27].

To purify the rat pancreatic somatostatin receptor, purified pancreatic acinar plasma membranes were prebound with S28 and solubilized with the detergent CHAPS. After gel filtration on Sephadex G-25-SF column, S28–receptor complexes were recovered in the void volume as previously described [24,25] and incubated with immobilized anti-[S28 (1–14)] antibody for 40 h at 4 °C. Bound-receptor complexes were eluted with an excess of S28 (1–14). SDS/PAGE analysis and silver staining of the eluted proteins revealed two major bands, one migrating at a molecular mass of 87 ± 2 kDa and another migrating at 45 kDa (Figure 1, lane 2). Whatever the experimental conditions, the 45 kDa band was always detected, showing that it represents a non-specifically associated protein. When BSA was used instead of ovalbumin, the 45 kDa band disappeared on behalf of a 67 kDa band, indicating that the 45 kDa band may correspond to ovalbumin (results not shown). The 87 kDa band was not detected when non-immune serum replaced the anti-[S28 (1–14)] serum (Figure 1, lane 1) or when pancreatic membranes were incubated in the absence of S28 (Figure 1, lane 3), demonstrating the specificity of the reaction. A similar molecular mass was observed for the somatostatin receptor after cross-linking of ^{125}I-[Tyr^{15}]S28 to pancreatic membranes (results not shown). In addition, we previously detected a 85–90 kDa protein after cross-linking of other iodinated somatostatin analogues to pancreatic somatostatin receptors in membranes [25,34] or soluble extracts [24,25].

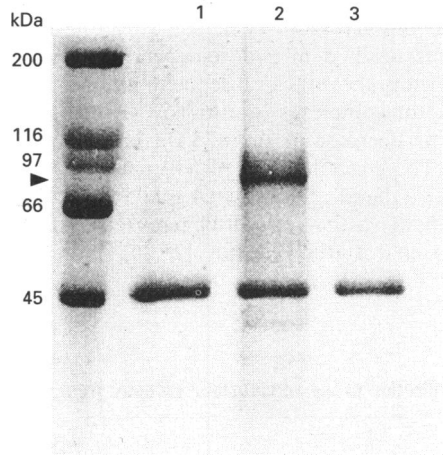

Figure 1 SDS/PAGE analysis of the purified somatostatin receptor

Solubilized somatostatin–receptor complexes from S28-treated (lanes 1 and 2) or untreated (lane 3) pancreatic membranes (7 mg/sample) were incubated with immobilized anti-[S28 (1–14)] antibodies (lanes 2 and 3) or non-immune antiserum (lane 1). Bound proteins were eluted with S28 (1–14), concentrated and loaded on a SDS/5–15% gradient polyacrylamide gel and the proteins were stained with silver as described in the Experimental section. Markers are shown in the first lane and their size is given in kDa.

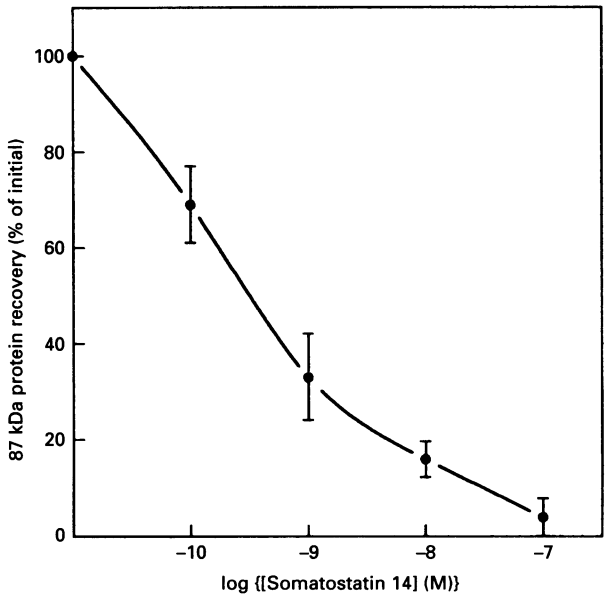

Figure 2 Concentration dependence of the effect of S14 on the 87 kDa protein recovery in immunoaffinity-purified somatostatin receptor preparations

Pancreatic membranes were incubated with 0.1 nM S28 (control) and increasing concentrations of S14. Membranes were solubilized, incubated with immobilized anti-[S28 (1–14)] antibody and proteins were eluted with S28 (1–14) as described in the Experimental section. Eluted material was concentrated, electrophoresed and silver stained. Quantification of the 87 kDa band was performed by image analysis as described in the Experimental section. Values for 87 kDa band recovery are expressed as percentage of the control. Each point is the mean ± S.E.M. of three separate experiments.

These results suggest that the 87 kDa band corresponds to the purified somatostatin receptor.

To verify that the appearance of the 87 kDa band is related to the binding of S28 to membranes, pancreatic membranes were exposed to S28 and increasing concentrations of S14 which was able to compete with S28 for binding to somatostatin receptors with a high affinity [26]. Then somatostatin receptors were purified. This resulted in a dose-dependent inhibition of S28 binding to membranes and a subsequent inhibition of the binding of S28–receptor complexes to anti-[S28 (1–14)] antibody as reflected by the decrease in the 87 kDa band staining intensity (Figure 2). The concentration of S14 causing 50% inhibition of the 87 kDa band staining (0.4 ± 0.05 nM; mean ± S.E.M., $n = 3$) is similar to the previously reported affinity of S14 for pancreatic somatostatin receptor [25,26], indicating that the binding of the 87 kDa protein to the S28 (1–14) antibody closely parallelled the binding of S28 to the membrane receptor.

On the basis of the estimated protein content of the purified receptor, the binding capacity of the purified receptor which could be calculated, assuming one somatostatin-binding site/receptor protein and a molecular mass of 87 kDa for the somatostatin receptor, was determined as 5.5 nmol/mg. This corresponds to a 9400-fold purification of the somatostatin receptor over the membrane-bound receptor (Table 1).

Tyrosine phosphatase activity of the immunoaffinity eluate

In normal and tumoural pancreatic cells, we and others demonstrated that somatostatin or its analogues stimulated a membrane tyrosine phosphatase activity which dephosphorylated phosphorylated EGF receptors by a mechanism involving receptor occupancy [20–22]. To demonstrate further that activated somatostatin receptors mediate somatostatin-induced tyrosine phosphatase stimulation, we tested the tyrosine phosphatase activity of immunoaffinity chromatography eluates containing purified somatostatin receptors. Two phosphotyrosyl substrates were used: an artificial, ^{32}P-poly(Glu,Tyr), and a more physiological substrate, ^{32}P-EGF receptor. These two substrates have been used previously to characterize various tyrosine phosphatase activities [35,36].

A431 cell membrane fractions were subjected to *in vitro* phosphorylation using [γ-^{32}P]ATP. After SDS/PAGE and autoradiography, the major [^{32}P]phosphate-labelled band was the autophosphorylated EGF receptor with an apparent molecular mass of 170 kDa (Figure 3, lane 1). Incubation of phosphorylated A431 cell membranes with increasing concentrations of immunoaffinity-purified proteins derived from S28-treated membranes resulted in an increase of the dephosphorylation of the ^{32}P-EGF-receptor band (Figure 3, lanes 2–4). The dephosphorylation of the EGF-receptor band reached 48 ± 3% (mean ± S.E.M., $n = 3$) in the presence of 100 ng of immunoaffinity-purified proteins. This suggested that purified somatostatin receptor preparations exhibited a tyrosine phosphatase activity that dephosphorylated ^{32}P-EGF receptor. When 5 mM of the sulphydryl compound dithiothreitol, a known stimulator of tyrosine phosphatases [37], was added in the assay the dephosphorylation of the EGF-receptor band exhibited by immunoaffinity-purified proteins was increased by 42 ± 4% (mean ± S.E.M., $n = 2$). Furthermore, the tyrosine phosphatase activity of the immunoaffinity eluate was suppressed by addition to the assay of 1 mM orthovanadate, the potent inhibitor of tyrosine phosphatases [37] (results not shown). When identical experiments were performed on immunoaffinity-purified material (100 ng) derived from membranes which had not been treated with S28, this material exhibited a low tyrosine

Table 1 Purification of the somatostatin receptor from rat pancreatic membranes

Sample	Total protein (mg)	Total binding (pmol)	Specific activity* (pmol/mg)	Recovery (%)	Purification (-fold)
Purified membranes	7	4	0.58	100	1
Solubilized membranes	3.9	2.1	0.55	52	0.95
Purified receptor	0.09×10^{-3}†	0.49	5470	6.9	9400

* Calculated from binding experiments carried out with ^{125}I-[Tyr^{15}]S28.

† Determined by image analysis of silver-stained SDS/PAGE gels using molecular-mass markers as standards.

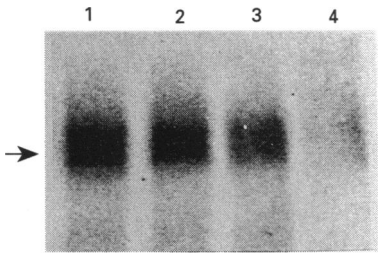

Figure 3 Dephosphorylation of ^{32}P-EGF-receptor by immunoaffinity-purified somatostatin receptor preparations

EGF receptors from A431 cell membranes were autophosphorylated with [γ-^{32}P]ATP and EGF as described in the Experimental section. Equal amounts of ^{32}P-labelled EGF receptors were incubated for 10 min at 30 °C with elution buffer (lane 1) or with increasing concentrations of immunoaffinity-purified somatostatin receptor preparations (5, 30 and 97 ng, lanes 2, 3 and 4 respectively) derived from S28-treated membranes. Proteins were separated by SDS/PAGE followed by autoradiography. The arrowhead indicates the position of autophosphorylated EGF receptor.

Table 2 S28 stimulation of tyrosine phosphatase activity in immunoaffinity-purified protein fractions

Solubilized somatostatin–receptor complexes from S28-treated (with) and untreated (without) pancreatic membranes (7 mg/sample) were incubated with immobilized anti-[S28 (1–14)] antibody. The bound material was washed (washing) and then eluted with S28 (1–14) (eluate) as described in the Experimental section. Material in washing and eluted fractions was concentrated and tyrosine phosphatase assays were performed using ^{32}P-poly(Glu,Tyr) as substrate. Data represent the mean ± S.E.M. of three (eluate) or five (washing) experiments performed in duplicate. Statistical significance was determined using Student's paired *t* test, for differences between values for without versus with S28 (*$P < 0.05$).

	Tyrosine phosphatase activity (unit/mg of protein)	
Fractions	Without S28	With S28
Washing	0.03 ± 0.01	0.05 ± 0.01*
Eluate	3 ± 0.4	8.6 ± 1.4*

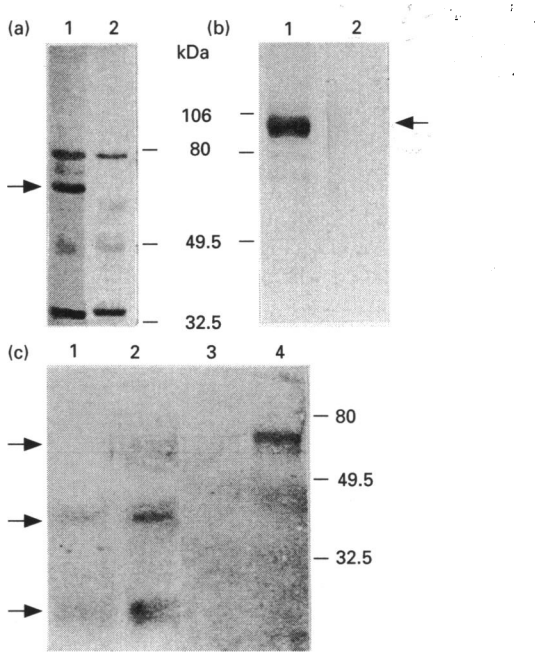

Figure 4 Detection of tyrosine phosphatase in immunoaffinity-purified somatostatin receptor preparations by Western blotting

(**a**) Solubilized pancreatic membranes (70 μg) were electrophoresed on a 10% (w/v) polyacrylamide gel, transferred to nitrocellulose and blotted with anti-(tyrosine phosphatase SHPTP1) antibodies (lane 1) or with preimmunoserum (lane 2) as described in the Experimental section. (**b**) Purified human recombinant glutathione S-transferase–SHPTP1 fusion protein (50 ng) was preincubated with (lane 2) or without (lane 1) the peptide (0.5 mM) used for immunization overnight at 4 °C and then blotted with anti-SHPTP1 antibodies. (**c**) Purified pancreatic membranes (15 mg) were incubated with (lanes 2 and 4) or without 2 nM S28 (lanes 1 and 3). Solubilized somatostatin–receptor complexes were incubated with immobilized anti-[S28 (1–14)] antibody and the bound material was washed and eluted with S28 (1–14) as described in the Experimental section. Material in washing (lanes 1 and 2) and eluted (lanes 3 and 4) fractions were concentrated and electrophoresed on a 10% polyacrylamide gel. The proteins were transferred to nitrocellulose and blotted with anti-SHPTP1 antibodies. Protein molecular-mass markers are indicated. Immunoreactive proteins (**a**: 66; **b**: 92; **c**: 66, 45, 25 kDa) are indicated by arrows.

phosphatase activity since dephosphorylation of the ^{32}P-EGF receptor reached about 10–15% (results not shown).

When ^{32}P-poly(Glu,Tyr) was used as substrate, immunoaffinity-purified preparations containing somatostatin receptors dephosphorylated ^{32}P-poly(Glu,Tyr) in a time-dependent manner, with a linear reaction for 15 min at 30 °C (results not shown). The specific enzyme activity in purified material containing somatostatin receptors was estimated to be 2.7 ± 0.2 ($P < 0.05$)-fold above the level detected in purified material derived from untreated membranes (Table 2). Such an increase was not seen when non-immune serum was used instead of immune serum (results not shown). Tyrosine phosphatase activity was also detected in washing fractions and the specific enzyme activity observed in the fractions derived from treated membranes was increased by 1.8 ± 0.2 ($P < 0.05$)-fold above that detected in fractions derived from untreated membranes, indicating that the enzyme material specifically bound to the immunoaffinity column might have been partially removed by washing buffer (Table 2). The specific activity of the enzyme in immunoaffinity-purified material corresponded to an enrichment of about 50-fold over that observed in solubilized material (specific activity: 0.12 ± 0.04 nmol/min per mg). Tyrosine phosphatase activity of immunoaffinity-purified material was inhibited by 59 ± 4% (mean ± S.E.M., $n = 3$) by 0.1 mM orthovanadate and increased by 110 ± 10% (mean ± S.E.M., $n = 2$) over controls in the presence of 5 mM dithiothreitol.

All these results indicated that the enzyme activity exhibited by immunoaffinity-purified material is that of a tyrosine phosphatase and that stimulation of membrane receptors by somatostatin resulted in activation of a tyrosine phosphatase which co-purified with somatostatin receptors.

We previously demonstrated that, in rat pancreatic acinar cells, somatostatin-stimulated membrane tyrosine phosphatase activity eluted at an apparent molecular mass of 70 kDa and the h.p.l.c.-purified 70 kDa protein reacted with antibodies directed towards the conserved domain of tyrosine phosphatase as a 67 kDa component associated with lower-molecular-mass proteolytic fragments [22]. Among the recently cloned tyrosine phosphatases, the 68 kDa phosphotyrosine phosphatase SHPTP1, possessing two Src homology region 2 domains (SH2), has the potential to promote intermolecular protein–protein interactions and to associate with membrane proteins [31]. To test whether tyrosine phosphatase activity detected in immunoaffinity-purified material is immunologically related to SHPTP1, we generated a rabbit polyclonal antibody against a synthetic peptide corresponding to 15 amino acids of the carboxyl region common to human

SHPTP1 and rat SHPTP1. Immunoblotting of the rat pancreatic membranes showed that the antibodies specifically recognized a 66 kDa protein (Figure 4a, lane 1) whereas the preimmune serum did not (Figure 4a, lane 2). The molecular mass of the protein recognized by the antibodies closely corresponded to that of SHPTP1. The antibodies also recognized the purified human recombinant glutathione S-transferase–SHPTP1 fusion protein containing the full-length human SHPTP1 as a major band at the predicted size of 92 kDa (Figure 4b, lane 1). This immunoreactive band was eliminated when Western blotting was performed in the presence of an excess of the peptide used to raise antibodies (Figure 4b, lane 2).

Immunoblotting using anti-SHPTP1 antibodies was next carried out with eluted proteins from the immunoaffinity column. As observed in Figure 4, the antibodies reacted with a band of 66 ± 2 kDa which was present in the purified material derived from S28-treated membranes (Figure 4c, lane 4) but not in purified material derived from untreated membranes (Figure 4c, lane 3) or after Western blotting with a non-immune serum (results not shown). In addition, a major band at 45 kDa and a faint band at 25 kDa, which probably corresponded to proteolytic fragments, were detected in washing material from S28-treated membranes (lane 2) but not in material derived from untreated membranes (lane 1).

To estimate the amount of tyrosine phosphatase in the immunopurified material, tyrosine phosphatase activity of the eluted material was compared with that of the purified human recombinant SHPTP1 enzyme, performed in the presence of ^{32}P-poly(Glu,Tyr) substrate. An estimated amount of 15 ± 7 ng and 3 ± 1 ng (mean $\pm$ S.E.M., $n = 3$) of tyrosine phosphatase was detected in the immunopurified material derived from 7 mg of S28-treated membranes and untreated membranes respectively. The same range of tyrosine phosphatase concentration can be estimated in the immunopurified material by comparing the intensity of the 66 kDa band in this material with that of known concentrations of purified recombinant SHPTP1 after immunoblotting with anti-SHPTP1 antibody (results not shown). These results indicate that about 0.2 pmol of enzyme co-purified with 1 pmol of somatostatin receptor.

Anti-SHPTP1 antibodies immunoprecipitate somatostatin receptors

The above information raised the possibility that the tyrosine phosphatase we have detected with immunopurified somatostatin receptors associates with the ligand-activated somatostatin receptor at the membrane level. Therefore a SHPTP1-immunoprecipitate from prelabelled membranes should have somatostatin-binding activity. To test this point, membrane somatostatin receptors were prelabelled with ^{125}I-[Tyr^3]SMS, solubilized and subjected to immunoprecipitation with the anti-SHPTP1 antibodies. The amount of specific ^{125}I-[Tyr^3]SMS binding present in immunoprecipitates was then measured. Anti-SHPTP1 immunoprecipitated specific labelled somatostatin receptors from solubilized membranes, as indicated by the significant increase in the level of specific binding of ^{125}I-[Tyr^3]SMS in the anti-SHPTP1 immunoprecipitates when compared with non-immune controls (Figure 5a). This represented approx. 2% of the soluble prelabelled somatostatin receptors. To determine whether SHPTP1 associated with somatostatin receptors in resting membranes in the absence of ligand activation of somatostatin receptors, non-prelabelled pancreatic membranes were solubilized and immunoprecipitated with anti-SHPTP1 serum. Immunoprecipitates were then subjected to somatostatin-binding assay using ^{125}I-[Tyr^3]SMS as radioligand. Anti-SHPTP1 antibodies immunoprecipitated functional somatostatin receptors from solubilized

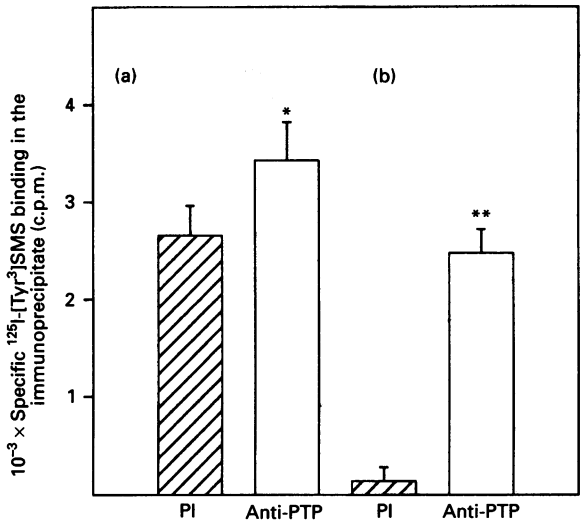

Figure 5 Anti-SHPTP1 antibodies immunoprecipitate occupied or unoccupied somatostatin receptors

(**a**) Pancreatic membranes (300 μg) were prelabelled with 1 nM ^{125}I-[Tyr^3]SMS in the presence or absence of 1 μM SMS, then solubilized and soluble ^{125}I-[Tyr^3]SMS–receptor complexes were immunoprecipitated with anti-SHPTP1 antibodies (antiPTP) or with preimmune control serum (PI) as described in the Experimental section. Specific ^{125}I-[Tyr^3]SMS binding was detected in the immunoprecipitates. (**b**) Soluble somatostatin receptors from pancreatic membranes (300 μg) were immunoprecipitated with anti-SHPTP1 antibodies (antiPTP) or with preimmune control serum (PI). The presence of somatostatin receptors in the immunoprecipitates was detected using the ^{125}I-[Tyr^3]SMS binding assay as described in the Experimental section. Values are presented as the amount of specific ^{125}I-[Tyr^3]SMS binding (c.p.m.) and are the mean $\pm$ S.E.M. of three different experiments done in duplicate. The statistical significance between preimmune controls and antiPTP is indicated by $^*P < 0.05$, $^{**}P < 0.01$.

pancreatic membranes. This was shown by a significant increase in the level of specific binding of ^{125}I-[Tyr^3]SMS to somatostatin receptors in the anti-SHPTP1 immunoprecipitates compared with that observed in preimmune controls (Figure 5b). This represented a specific precipitation of approx. 5% of active soluble somatostatin receptors. These findings are consistent with the hypothesis that SHPTP1 or an SHPTP1-related protein complexes with somatostatin receptors and that this association is observed in the resting state.

DISCUSSION

This report demonstrates that pancreatic acinar somatostatin receptor complexes purified from somatostatin-treated membranes contain a tyrosine phosphatase that dephosphorylates phosphorylated EGF receptor and poly(Glu,Tyr) and is recognized by anti-SH2-containing tyrosine phosphatase SHPTP1 antibodies as a 66 kDa immunoreactive protein. To provide evidence that such a tyrosine phosphatase co-purified with activated somatostatin receptors, we took advantage of the fact that pancreatic acinar membranes exhibit somatostatin receptors which display high affinity for the two somatostatin molecules, S14 and S28 [25], to purify S28–receptor complexes from this target by immunoaffinity chromatography using anti-[S28 (1–14)] antibodies. This purified protein was identified as somatostatin receptor by virtue of the ability of anti-[S28 (1–14)] antibody to specifically immunopurify solubilized S28-occupied receptors but not S14-occupied or free unoccupied receptors. In addition, we observed that S14 inhibited the ability of the antibody to specifically retain S28-bound receptors with its expected affinity for pancreatic somatostatin receptors. These observations, to-

gether with the finding that the same apparent molecular mass was previously identified by cross-linking studies of pancreatic membrane or solubilized bound-somatostatin receptors using iodinated S14 or S28 analogue probes [24,25,34], suggest that the 87 kDa protein represents the pancreatic somatostatin receptor. A similar apparent molecular mass has been identified recently in GH_4C_1 rat pituitary tumour cells [11,38] and a gastric mucosal cell line [10] for purified somatostatin receptors. However, it is different from the molecular mass of 60 kDa reported for the somatostatin receptor in brain, adrenal cortex and the AtT-20 and GH_3 pituitary-derived cells [39]. Until now, it is not known which receptor subtype(s) correspond(s) to the 87 kDa protein. However, rat pancreatic acinar cells highly express SSTR2 mRNA [40] and have somatostatin receptors with similar pharmacological characteristics to SSTR2 [13,25], suggesting that the SSTR2 receptor subtype could be a candidate for the 87 kDa protein.

The present studies show that a tyrosine phosphatase activity was found to co-purify with somatostatin–receptor complexes. This enzyme activity was detected using phosphorylated substrates including poly(Glu,Tyr) and EGF receptor, previously shown to be potential substrates for tyrosine phosphatases [22,23,35,36]. The enzyme activity shared characteristics with tyrosine phosphatases: it was stimulated by the reducing agent dithiothreitol and inhibited by orthovanadate. It was specifically detected in the immunopurified material containing somatostatin receptors. The extent of the increase of tyrosine phosphatase activity by S28 in the purified material was similar to that previously reported for platelet-derived growth factor or platelet-activating factor stimulation of receptor-associated tyrosine kinase in anti-receptor immunoprecipitates [41] or anti-phosphotyrosine affinity eluates [42]. Furthermore, we were able to detect a 66 kDa immunoreactive protein to anti-(SH2-containing tyrosine phosphatase SHPTP1) antibodies in immunopurified material containing somatostatin receptors, but we failed to detect such a protein in the absence of somatostatin receptor in the purified material. This suggests that the presence of the enzyme is related to the presence of somatostatin receptors.

Finally, the observation that anti-SHPTP1 antibodies immunoprecipitated specific ^{125}I-[Tyr^3]SMS binding from prelabelled pancreatic membranes raises the possibility that SHPTP1 is associated with somatostatin receptors after ligand activation. The demonstration that anti-SHPTP1 antibodies immunoprecipitated functional somatostatin receptors derived from unstimulated membranes indicates that SHPTP1 is able to associate with unoccupied somatostatin receptor and suggests that the somatostatin receptor–SHPTP1 complexes pre-exist at the cell membrane even in the absence of ligand. Whether SSTR2 is the somatostatin receptor subtype associated with the tyrosine phosphatase is an important issue that we are currently addressing. The recent demonstration that, in NIH3T3 cells expressing SSTR2 somatostatin receptor subtype, somatostatin activated a membrane tyrosine phosphatase [40], suggests that SSTR2 could be able to associate with SHPTP1.

The low purification factor of the tyrosine phosphatase contrasts with that of the somatostatin receptor and may be related to the loss of the protein and/or its activity during the purification procedure. The presence of 5-fold more somatostatin receptor molecules than that of tyrosine phosphatase in purified material and the detection of specific tyrosine phosphatase activity in corresponding washing fractions are consistent with this hypothesis. Indeed, tyrosine phosphatases are well known to be highly sensitive to proteolytic degradation which generates a soluble and smaller molecule [22,23,35]. This assumption is also supported by the presence in washing fractions of a 45 kDa immunoreactive protein which probably represents a proteolytic fragment of the 66 kDa form as previously observed after immunoblot analysis of partially purified pancreatic tyrosine phosphatase [22]. Another possibility would be that the purified receptor preparations contain several receptor subtypes, only one of which co-purifies with the tyrosine phosphatase or that only a small amount of receptors are associated with the tyrosine phosphatase. This is consistent with the observation that a low level of somatostatin receptors was detected in anti-SHPTP1 immunoprecipitates. While the existence of a factor that co-purifies with somatostatin receptors and stimulates a tyrosine phosphatase endogenous to phosphorylated substrates cannot be excluded. The fact that the [^{32}P]P_i released by the phosphorylated substrate poly(Glu,Tyr) alone, in the absence of eluted proteins, was not sensitive to dithiothreitol stimulation and vanadate inhibition precludes the presence of such an endogenous tyrosine phosphatase in the substrate preparation.

Evidence in support of the role of tyrosine phosphatases in signal transduction has been accumulated [43]. Our observation that a 66 kDa tyrosine phosphatase immunoreactive to anti-SHPTP1 antibodies protein co-purified with activated somatostatin receptors and that anti-SHPTP1 antibodies immunoprecipitated somatostatin receptors suggests a direct link between the physiological function of this enzyme and the somatostatin receptor. The physiological role of SHPTP1 is not known but this enzyme has the potential to participate in signal transduction pathways through its SH2 domains, which are able to promote protein–protein interactions via binding to specific tyrosyl phosphorylated substrates [31]. The demonstration that SHPT1 forms high-affinity complexes with the activated EGF receptor [44] and dephosphorylates activated growth factor receptors *in vivo* [45,46] raises the possibility that SHPTP1 has a role in the negative control of growth factor receptor-mediated signals. Such a role has been recently demonstrated in interleukin-3-dependent haematopoietic cells where overexpression of SHPTP1, designated as HCP (haematopoietic cell phosphatase), suppresses growth in these cells [46].

While the precise role of tyrosine phosphatase in the action of somatostatin is unresolved, evidence has begun to accumulate suggesting that the stimulation of a tyrosine phosphatase activity is one of the signal transduction pathways promoted by G-protein-coupled receptors that negatively regulate growth [40,47]. Somatostatin or analogues inhibit cell proliferation stimulated by growth factors [4,48,49] and displayed the same order of potency to inhibit somatostatin binding and cell growth and to stimulate tyrosine phosphatase activity [21,22,40]. Recent studies on cells overexpressing tyrosine phosphatases suggest that these enzymes may act as counter-regulators of mitogenic tyrosine kinases [43]. In conjunction with the observation that somatostatin reversed the stimulatory effect of EGF on the phosphorylation of the EGF receptor and endogenous proteins [21,50], our results suggest a possible role for the SHPTP1 or SHPTP1-related tyrosine phosphatase in an early signal transduction pathway mediated by the somatostatin receptor and involved in the antiproliferative effect of somatostatin.

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