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Carboxyl-terminal multi-site phosphorylation regulates internalization and desensitization of the human sst_2 somatostatin receptor ☆

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ABSTRACT

The somatostatin receptor 2 (sst_2) is the pharmacological target of somatostatin analogs that are widely used in the diagnosis and treatment of human neuroendocrine tumors. We have recently shown that the stable somatostatin analogs octreotide and pasireotide (SOM230) stimulate distinct patterns of sst_2 receptor phosphorylation and internalization. Like somatostatin, octreotide promotes the phosphorylation of at least six carboxyl-terminal serine and threonine residues namely S341, S343, T353, T354, T356 and T359, which in turn leads to a robust receptor endocytosis. Unlike somatostatin, pasireotide stimulates a selective phosphorylation of S341 and S343 of the human sst_2 receptor followed by a partial receptor internalization. Here, we show that exchange of S341 and S343 by alanine is sufficient to block pasireotide-driven internalization, whereas mutation of T353, T354, T356 and T359 to alanine is required to strongly inhibited both octreotide- and somatostatin-induced internalization. Yet, combined mutation of T353, T354, T356 and T359 is not sufficient to prevent somatostatin-driven β-arrestin mobilization and receptor desensitization. Replacement of all fourteen carboxyl-terminal serine and threonine residues by alanine completely abrogates sst_2 receptor internalization and β-arrestin mobilization in HEK293 cells. Together, our findings demonstrate for the first time that agonist-selective sst_2 receptor internalization is regulated by multi-site phosphorylation of its carboxyl-terminal tail.

1. Introduction

The somatostatin receptor sst_2 is highly expressed at the plasma membrane of human tumors including pancreatic, gastrointestinal and pulmonary neuroendocrine tumors, pituitary adenomas, breast carcinomas, meningiomas, neuroblastomas, medulloblastomas, pheochromocytomas, and paragangliomas. This is the molecular basis for clinical application of stable somatostatin analogs for tumor imaging and tumor therapy. A number of metabolically stable somatostatin analogs have been synthesized two of which, octreotide and lanreotide, were approved for clinical use. Octreotide and lanreotide bind with high sub-nanomolar affinity to sst_2 only, have moderate affinity to sst_3 and sst_5 and show very low or absent binding to sst_1 and sst_4 (Colao et al., 2010).

In clinical practice, octreotide and lanreotide are used as first choice medical treatment of neuroendocrine tumors such as GH-secreting adenomas and carcinoids (Donangelo and Melmed, 2005; Oberg et al., 2010). Octreotide initially controls symptoms caused by hormonal overproduction in about 90% of carcinoid patients. After 1 year of treatment, however, some 50% of patients show an escape of response (Oberg, 2005; Asnacios et al., 2008). In contrast, octreotide can normalize GH levels for prolonged periods in 65% of acromegalic patients (Donangelo and Melmed, 2005). Recently, the novel multireceptor somatostatin analog, pasireotide (SOM230), has been synthesized (Bruns et al., 2002). Pasireotide is a cyclohexapeptide, which binds with high affinity to all somatostatin receptors except to sst_4 (Lewis et al., 2003).

We have recently uncovered that octreotide and pasireotide stimulate distinct agonist-selective patterns of sst_2 somatostatin receptor phosphorylation and internalization (Poll et al., 2010; Lesche et al., 2009; Kliewer et al., 2012). Like somatostatin, octreotide promotes the phosphorylation of at least six carboxyl-terminal serine and threonine residues namely S341, S343, T353,

Abbreviations: CHO, chinese hamster ovarian cells; GH, growth hormone; GPCR, G-protein coupled receptor; HEK293, human embryonic kidney 293 cells; SS-14, somatostatin-14; sst, somatostatin receptor.

☆ Phosphorylation of multiple carboxyl-terminal sites within the including S341, S343, T353, T354, T356 and T359 directly regulates agonist-driven internalization and desensitization of the human sst_2 somatostatin receptor.

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T354, T356 and T359, which in turn leads to a robust sst_2 receptor endocytosis (Nagel et al., 2011; Kao et al., 2011). Unlike somatostatin, pasireotide fails to induce a substantial phosphorylation or internalization of the sst_2 receptor (Nagel et al., 2011). These findings suggest that agonist-driven phosphorylation may facilitate sst_2 receptor endocytosis. However, earlier studies have failed to establish a causal relationship between phosphorylation and internalization for the rat sst_2 receptor (Liu et al., 2008). Here, we have constructed a series of phosphorylation-deficient mutants and examined the contribution of individual phosphorylation events to agonist-dependent regulation of the human sst_2 receptor.

2. Materials and methods

2.1. Reagents, plasmids and antibodies

Pasireotide and octreotide were kindly provided by Dr. Herbert Schmid (Novartis, Basel, Switzerland). SS-14 was obtained from Bachem (Weil am Rhein, Germany). DNA for HA-tagged 19S/T-A, 14S/T-A, 6S/T-A, 4T-A and 2S-A mutants of the human sst_2 receptor was generated via artificial gene synthesis and cloned into pcDNA3.1 by imaGenes (Berlin, Germany). The human HA-tagged sst_2 receptor was obtained from UMR cDNA Resource Center (Rolla, MO). The phosphorylation-independent rabbit monoclonal anti-sst_2 antibody {UMB-1} (Epitomics, Burlingame, CA) and the phosphosite-specific sst_2 antibodies anti-pS341/pS343 {3157}, anti-pT353/pT354 {0521}, anti-pT356/pT359 {0522} and the rabbit polyclonal anti-HA antibody were generated and extensively characterized as previously described (Poll et al., 2010; Fischer et al., 2008). Antibodies {0521} and {0522} were generated against a peptide containing pT353, pT354, pT356 and pT359 and subsequently affinity purified against peptides containing either pT353 and pT354 {0521} or pT356 and pT359 {0522} (Poll et al., 2010, 2011; Nagel et al., 2011).

2.2. Cell culture and transfection

Human embryonic kidney HEK293 cells were obtained from the German Resource Centre for Biological Material (DSMZ, Braunschweig, Germany). HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum. Cells were transfected with plasmids encoding for wild-type or mutant sst_2 receptors using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA). Stable transfectants were selected in the presence of 400 μg/ml G418. Stable cells were characterized using radioligand-binding assays, Western blot analysis, surface ELISA assay and immunocytochemistry as described previously (Tulipano et al., 2004; Pfeiffer et al., 2001). All mutants tested were present at the cell surface, expressed similar amounts of receptor protein and had similar affinities for SS-14 as the wild-type receptor. The level of receptor expression was ~800 fmol/mg membrane protein for all experiments using stably transfected cells. The level of receptor expression was between 1500 and 2000 fmol/mg membrane protein for all experiments using transiently transfected cells.

2.3. Analysis of receptor internalization by confocal microscopy

HEK293 cells satbly expressing HA-tagged human sst_2 receptors were grown on poly-L-lysine-coated coverslips overnight. When indicated cells were transiently transfected with 1 μg GRK2 plasmid DNA per well containing 100,000 cells using TurboFect™ (Fermentas) according to the instructions of the manufacturer. After the appropriate treatment with either 1 μM SS-14, 1 μM Octreotide or 10 μM Pasireotide at 37 °C, cells were fixed with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer (pH 6.9) for 30 min at room temperature and washed several times. Specimens were permeabilized and then incubated with anti-sst_2 {UMB-1} antibody followed by Alexa488-conjugated secondary antibody (Amersham, Braunschweig, Germany). Specimens were mounted and examined using a Zeiss LSM510 META laser scanning confocal microscope (Lesche et al., 2009).

2.4. Quantification of receptor internalization by ELISA

Receptor internalization was quantified using a linear surface receptor ELISA that has been characterized extensively (Poll et al., 2010; Lesche et al., 2009; Nagel et al., 2011; Pfeiffer et al., 2001). Equal numbers of stably transfected HEK293 cells expressing HA-tagged human sst_2 receptors were seeded onto poly-L-lysine-treated 24-well plates (200.000 cells per well). The next day, cells were preincubated with 1 μg/ml anti-HA antibody for 2 h at 4 °C. After the appropriate treatment with SS-14 (1 μM) at 37 °C, cells were fixed and incubated with peroxidase-conjugated anti-rabbit antibody overnight. After washing, plates were developed with ABTS solution and analyzed at 405 nm using a microplate reader. When indicated cells were transiently transfected with 1 μg GRK2 plasmid DNA per well using TurboFect™ (Fermentas) 24 h later, cells were preincubated with 1 μg/ml anti-HA antibody and treated as described above. Statistical analysis was carried out with unpaired *t*-test. *p*-Values of <0.05 were considered statistically significant.

2.5. Western blot analysis

HEK293 cells stably expressing HA-tagged human sst_2 receptors were plated onto 60-mm dishes and grown to 80% confluence. After treatment with SS-14 at 37 °C, cells were lysed in detergent buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM disodium pyrophosphate, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) in the presence of protease and phosphatase inhibitors Complete mini and PhosSTOP (Roche Diagnostics, Mannheim, Germany) and centrifuged at 16,000*g* for 20 min at 4 °C. Glycosylated proteins were partially enriched using wheat germ lectin-agarose beads as described (Schulz et al., 2000; Mundschenk et al., 2003; Plockinger et al., 2008). Proteins were eluted from the beads using SDS-sample buffer for 20 min at 65 °C and then resolved on 8% SDS–polyacrylamide gels. After electroblotting, membranes were incubated with phosphosite-specific antibodies anti-pS341/pS343 {3157}, anti-pT353/pT354 {0521} or anti-pT356/pT359 {0522} at a concentration of 0.1 μg/ml followed by detection using enhanced chemiluminescence (Amersham). Blots were subsequently stripped and reprobed with anti-sst_2 antibody {UMB-1} to confirm equal loading of the gels.

2.6. β-Arrestin-EGFP mobilization assay

Untransfected HEK293 cells were seeded onto 35-mm glass-bottom culture dishes (Mattek, Ashland, MA). The next day, cells were transiently cotransfected with 0.2 μg β-arrestin-2-EGFP and 2 μg human sst_2 receptor or with a mixture of 0.2 μg β-arrestin-2-EGFP, 0.8 μg GRK2 and 1.2 μg human sst_2 receptor per dish containing 200,000 cells using TurboFect™. After 24 h, cells were transferred onto a temperature-controlled microscope stage set at 37 °C of a Zeiss LSM510 META laser scanning confocal microscope. Images were collected sequentially using single line excitation at 488 nm with 515–540-nm band pass emission filters. Saturating concentrations of SS-14 (1 μM) were applied directly into the culture medium immediately after the initial image was taken.