Identification of human SST2 Somatostatin Receptor domains involved in receptor internalization and signaling in pancreatic neuroendocrine tumours (P-NETs)

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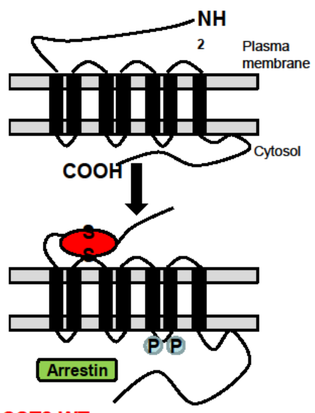
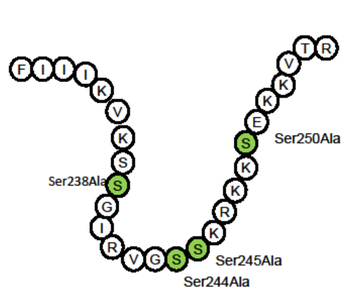
INTRODUCTION & OBJECTIVES

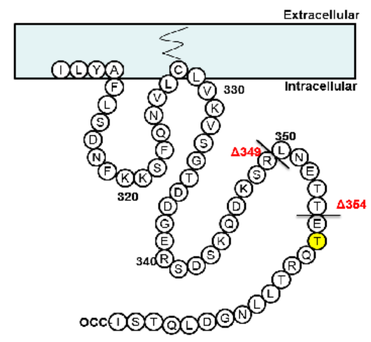
Somatostatin receptor family (SSTRs) consists of five different subtypes (SSTRs 1-5) belonging to the G-protein coupled receptor (GPCRs) family. After agonist binding to the receptor, the signaling is turned off (1) by phosphorylation of intracellular receptor domains and subsequent recruitment of arrestins, cytoplasmic proteins inducing signal termination (2).
80-90% of the GEP-NETs express SSTRs on the cell membrane and in particular SST2 is the mostly abundant subtype (3) representing the primary molecular target for somatostatin analogs in cancer therapy (4). Because somatostatin analogs are so important in the clinical management of neuroendocrine tumours, elucidating the role and mechanism of receptor phosphorylation in sst2 function is critical for understanding the regulation of this receptor under physiological conditions and during therapy.
The cytoplasmic regions within GPCR that are phosphorylated by GRKs and bind arrestins vary among different receptors (2). Previous studies show that upon agonist stimulation sst2 is phosphorylated exclusively in the carboxyl terminus (CT) and the third intracellular loop (IC3) (5; 6). In particular five serines and two threonines contained in the CT portion are phosphorylated upon somatostatin treatment (7; 8), however the IC3 loop residues remain to be elucidated.
In a pituitary cell line model, it has been demonstrated that the IC3 loop of SST5 is crucial for arrestin binding and receptor internalization (9) and identified two regions in the IC2 and IC3 required for the regulation of hormone secretion and proliferation (10).
Our goal was to identify in P-NETs the regulatory regions of the SST2 important for receptor desensitization and internalization and analyze the same domains to understand if are involved in the transduction of intracellular signals.

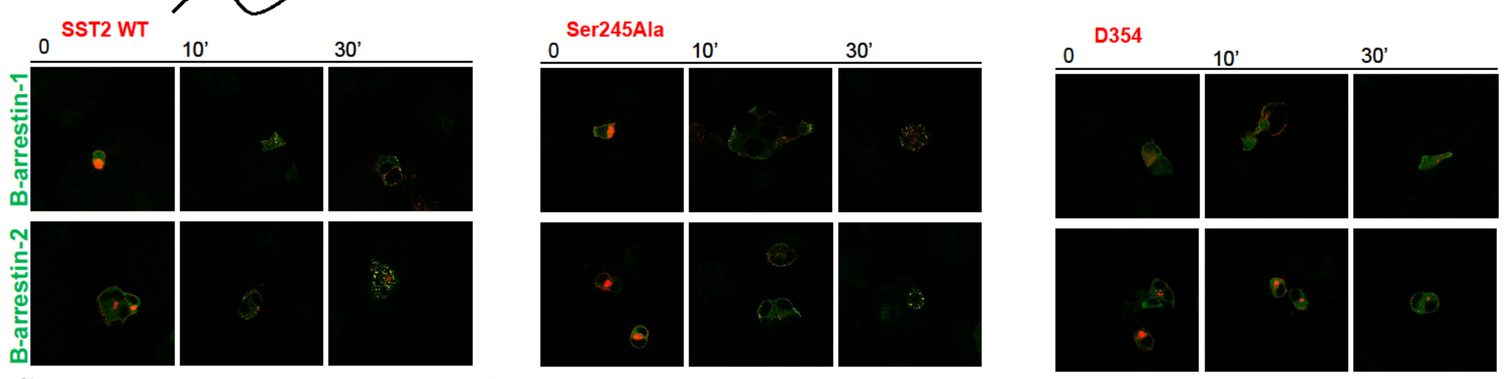
RESULTS

Characterization and identification of the SST2 structural domains involved in internalization process after somatostatin analog stimulation





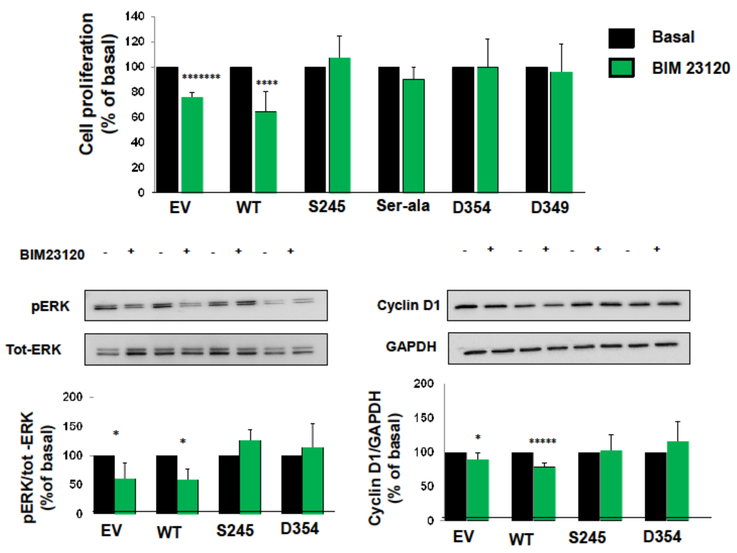




Results: After BIM-23120 stimulation, activated WT SST2 internalized and colocalized with B-arrestin-1 and 2. The same was observed for Ser245Ala IC3 mutant. Del354 SST2 CT mutant didn't associate with B-arrestins after BIM-23120 stimulation.

Methods: To generate the constructs, the wild type and mutated SST2 forms were subcloned into the pDsRed2-N1 expression vector. Truncated SST2 mutants were created by PCR using primers that introduce stop codons at suitable position ; pDsRed2-N1-D349 and D354 lacked respectively 20 and 15 amminoacids at the c-terminal tail.
Point mutations were introduced into SST2 third loop by PCR-based mutagenesis replacing serine 245 with alanine in the first single mutant (pDsRed2-N1Ser245Ala) and serines 237-238-244-245-250 with alanines in a multiple mutant (pDsRed2-N1Ser237-238-244-245-250Ala).
QGP-1 cells were transiently cotransfected with B-arrestin-1-YFP or B-arrestin-2-EGFP and SST2-DsRed2. 48 hours after transfection , cells were treated with saturating concentration of BIM23120 (10 nM) for the indicated times, fixed and analyzed at confocal microscopy.

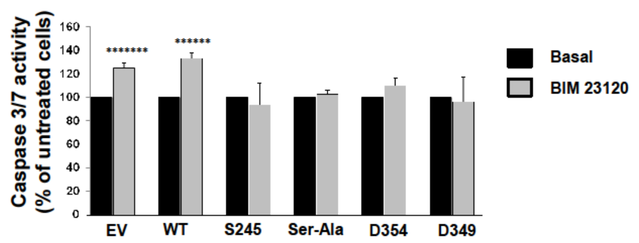
Effect of SST2 mutations on cell proliferation, ERK1/2 phosphorylation and cyclin D1 levels



Results: In QGP1 cells tranfected with SST2 WT, BIM-23120 induced an inhibition of proliferation. No effect on proliferation was mediated by receptor with mutations in CT and the IC3 portion. Representative immunoblot of ERK1/2 phophorylation and cyclin D1confirmed an expression level reduction in cells expressing SST2 WT. The effect was completely abolished with mutated SST2 receptors. Values represent mean (±SD.) *=p<0.05 ****=p<0.0001 *****=p<0.00001 *******=p<0.0000001 *vs* corresponding basal.

Methods: Cell proliferation was assessed by colorimetric measurement of 5-bromo-2'-deoxyuridine (BrdU) incorporation during DNA synthesis. Transfected cells were incubated with BIM-23120 10 nM for 48 hours and with BrdU for 2 hours to allow BrdU incorporation. For western blot analysis cells were transfected with empty vector EV or SST2 constructs for 48 hours and stimulated with BIM-23120 for 10 minutes to test ERK1/2 or 6 hrs for cyclin D1.

Effect of SST2 receptor mutations on apoptosis



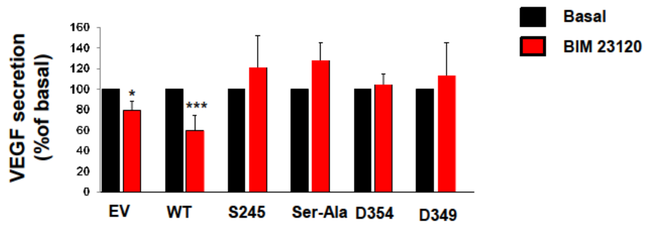
Results: As expected BIM-23120 induced apoptosis by activating SST2. The effect was completely abolished by all the mutants suggesting the importance of both the CT and IC3 SST2 portion in the pro-apoptotic effect mediation. Values represent mean (±SD.) ******=p<0.000001 *******=p<0.0000001 *vs* corresponding basal.

Methods: Caspase-3 enzymatic activity was measured using Apo-ONE homogenous caspase-3/7 assay (Promega) according to manufacturer instructions. After 48 hours of EV and SST2 constructs transfection, cells were treated with BIM-23120 10 nM for 48 hours.

Effect of SST2 receptor mutations on angiogenesis

Results: BIM-23120 reduced Vascular Endothelial Growth Factor (VEGF) release by activating SST2. The effect was completely abolished by all the mutants. Values represent mean (±SD.) *=p<0.05 ***=p<0.001 *vs* corresponding basal.

Methods: To analyze VEGF secretion, QGP1 cells were transfected with SST2 constructs for 48h, then the cells were treated with BIM-23120 10 nM in serum free medium for 72h at 37°C. Collected supernatants were used to measured VEGF concentration with ELISA kit (Invitrogen, Camarillo, CA) according to manufacturer instructions.



CONCLUSIONS

Taken together these data suggest that:

1) SST2 receptor under somatostatin analog treatment internalizes and associates with both B-arrestin-1 and 2;
2) the carboxyl terminus (CT) region is crucial for B-arrestins/SST2 interaction and SST2 internalization;
3) the anti-proliferative intracellular signaling mediated by SST2 requires the integrity of both CT and third intracellular (IC3) domains;
4) the pro-apoptotic intracellular signaling mediated by SST2 requires the integrity of both CT and IC3 domains;
5) the anti-angiogenic effect mediated by SST2 requires the integrity of both CT and IC3 domains.

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