Filamin-A is required to mediate SST2 effects in pancreatic neuroendocrine tumours

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Abstract

Somatostatin receptor type 2 (SST2) is the main pharmacological target of somatostatin (SS) analogues widely used in patients with pancreatic neuroendocrine tumours (P-NETs), this treatment being ineffective in a subset of patients. Since it has been demonstrated that Filamin A (FLNA) is involved in mediating GPCR expression, membrane anchoring and signalling, we investigated the role of this cytoskeleton protein in SST2 expression and signalling, angiogenesis, cell adhesion and cell migration in human P-NETs and in QGP1 cell line. We demonstrated that FLNA silencing was not able to affect SST2 expression in P-NET cells in basal conditions. Conversely, a significant reduction in SST2 expression (−43 ± 21%, \(P<0.05\) vs untreated cells) was observed in FLNA silenced QGP1 cells after long term SST2 activation with BIM23120. Moreover, the inhibitory effect of BIM23120 on cyclin D1 expression (−46 ± 18%, \(P<0.05\) vs untreated cells), P-ERK1/2 levels (−42 ± 14%; \(P<0.05\) vs untreated cells), cAMP accumulation (−24 ± 3%, \(P<0.05\) vs untreated cells), VEGF expression (−31 ± 5%, \(P<0.01\) vs untreated cells) and in vitro release (−40 ± 24%, \(P<0.05\) vs untreated cells) was completely lost after FLNA silencing. Interestingly, BIM23120 promoted cell adhesion (+86 ± 45%, \(P<0.05\) vs untreated cells) and inhibited cell migration (−24 ± 2%, \(P<0.00001\) vs untreated cells) in P-NETs cells and these effects were abolished in FLNA silenced cells. In conclusion, we demonstrated that FLNA plays a crucial role in SST2 expression and signalling, angiogenesis, cell adhesion and cell migration in P-NETs and in QGP1 cell line, suggesting a possible role of FLNA in determining the different responsiveness to SS analogues observed in P-NET patients.
Introduction

Somatostatin (SS) is a ubiquitous peptide that physiologically inhibits hormone secretion from neuroendocrine cells. In particular, SS inhibits pituitary GH and TSH secretion and insulin, glucagon, and secretin secretion at gastro-entero-pancreatic level (Van Op den Bosch et al. 2009, Reubi & Schonbrunn 2013). These effects are mediated by five receptor subtypes (SST1-5) belonging to the G protein coupled receptors superfamily (GPCR) that differ in tissue distribution, affinity to ligands and mechanisms of action and regulation (Patel 1998, Lahlou et al. 2004). As demonstrated in cells transfected with different receptor subtypes and confirmed by the clinical use of selective agonists, SST2 and to lesser extent SST5 and SST3 appear to mediate most of the antisercretive and antiproliferative effects of SS (Reubi & Schonbrunn 2013).

Most neuroendocrine tumours (NETs) express SST2 and 5 that are targets for the long-acting SS analogues (Papotti et al. 2002, Reubi & Schonbrunn 2013, Theodoropoulou & Stallal 2013). Although in vivo and in vitro evidences support the antiproliferative action of SS in neuroendocrine cells, NETs show a variable biological response to SS analogues. In particular, octreotide LAR and lanreotide SR (with a preferential binding affinity for SST2) have been demonstrated to control both the symptoms and hormone secretion in 50–60% of patients with functioning NET. Moreover, SS analogues have been found to stabilise tumour mass in 30–50% of patients, with a significant reduction in tumour mass being observed in a minority of them (Rinke et al. 2009, Öberg 2012, Caplin et al. 2014), but the mechanisms involved in the resistance in non-responders patients being still unknown.

Several studies demonstrated that GPCRs expression and signalling are mediated by different cytoskeleton proteins, including filamin A (FLNA; Stossel et al. 2001, Huang et al. 2006). FLNA is a ubiquitous actin binding protein, involved in cell morphology and motility regulation, acts as a molecular scaffold for several proteins, including transmembrane proteins and signalling molecules. In addition, the relevance of FLNA in pituitary tumour responsiveness to pharmacological treatment has been demonstrated in PRL-secreting pituitary tumours, where FLNA plays a key role in dopamine receptor type 2 signalling and cell surface expression (Peverelli et al. 2012). Recently, FLNA/SST2 interaction has been found to play a critical role for SST2 stabilization and cell survival in human pancreatic tumour cells (BON) and in melanoma cell line (Najib et al. 2012) as well as in tumourous pituitary GH-secreting cells (Peverelli et al. 2014).

Moreover, the involvement of cytoskeletal actin-binding proteins in angiogenesis has been suggested as a target for anti-neovascular cancer therapy in vitro. In fact, a positive relationship between FLNA and vascular endothelial growth factor (VEGF) was found in patients with lung cancer (Uramoto et al. 2010), suggesting that FLNA is implicated in angiogenesis through links with VEGF. Interestingly, it has been demonstrated that VEGF pathway is overexpressed in neuroendocrine tumours (Öberg et al. 2013), this pathway being inhibited by somatostatin analogues (Villaume et al. 2010).

In addition, FLNA allows the cross-linking of actin filaments into a dynamic three dimensional structure and mediates the anchoring of several transmembrane proteins to the cortical actin. These FLNA functions are crucial for cell adhesion to extracellular matrix, cell locomotion and migration (Xu et al. 2010).

Aim of the present study was to investigate the role of FLNA in the regulation of SST2 stabilization, signalling, angiogenesis and cell motility in pancreatic neuroendocrine tumour cells.

Materials and methods

Immunohistochemistry

Immunohistochemistry experiments were performed on sections from 29 P-NETs retrieved from the archives of Pathology Unit of IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy. After dewaxing in Bioclear and rehydrating in ethanol, sections were pre-treated in a water bath set to 98°C in 0.01 M citrate buffer for 25 min. Mouse Monoclonal FLNA antibody (MAB1678, Millipore, Billerica, Massachusetts, USA, 1:600 dilution) and rabbit monoclonal SST2 antibody (ab134152, UMB-1; Abcam, Cambridge, UK; 1:200 dilution) were used, and antigen-antibody detection was performed with the MACH1 universal polymer detection kit (Biocare Medical, Concord, CA, USA). FLNA and SST2 immunoreactivities were graded according to the previously described immunohistochemical score (Peverelli et al. 2012) and analysed by an observer not connected with the project, evaluating at least 100 cells/section.

Pancreatic neuroendocrine tumour cell cultures

The study was approved by the local ethics committee. Informed consent was obtained from all subjects involved in the study. Human neuroendocrine cells were obtained
from 11 P-NETs that were enzymatically dissociated in DMEM containing 2 mg/ml collagenase (Sigma–Aldrich Corporate Headquarters) at 37 °C for 2 h. Dispersed cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics. Human pancreatic endocrine QGP-1 cell line (carcinoembryonic antigen (CEA)-producing human pancreatic islet cell carcinoma, JCRB0183, Japanese Homo Sapiens) was grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine and antibiotics. QGP1 cell line was cultured at 37 °C in 5% CO2 atmosphere.

FLNA silencing in pancreatic neuroendocrine cells

In order to obtain the best efficiency of FLNA silencing three different human FLNA silencer select pre-designed siRNAs (Ambion, Austin, TX, USA) were tested. Preliminary experiments to determine the optimal concentration of siRNAs and the kinetics of silencing of FLNA were performed. A negative control siRNA (C-siRNA), a non-targeting sequence without significant homology to the sequence of human, mouse or rat transcripts, was used in each experiment. Western blotting was performed in each experiment to control the FLNA expression level after silencing. FLNA gene silencing was performed in P-NET and QGP1 cells using species-specific human FLNA pre-designed siRNA (sense 5'-3', CCAACAAGCCCCAACAAGUUTT and antisense AACUUGUUUGCCUUGUGGTT) and Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instruction. Briefly, 400,000 cells were seeded into six-wells plates. Lipofectamine 2000 (5 ml) was diluted in Opti–MEM (Invitrogen) and FLNA siRNA, used at a concentration 30 nM, was also diluted in Opti-MEM, then added to diluted Lipofectamine 2000 Reagent (1:1 ratio) and incubated for 5 min at room temperature. Subsequently, QGP1 cells were incubated with the mixture for 72 h.

Western immunobLOTS of SST2, cyclin D1, pERK1/2 and VEGF proteins

QGP1 cells were treated with FLNA siRNA or C-siRNA and incubated at 37 °C with BIM23120 10 nM for 72 h to analyse SST2 and VEGF expression, 6 h for cyclin D1 and 10 min for pERK1/2. Cells were then lysed in lysis buffer in the presence of protease inhibitors. Proteins were separated on SDS/PAGE and transferred to a nitrocellulose filter. Antibodies raised against epitopes specific for SST2 (Santa Cruz; 1:200, sc365502, monoclonal antibody) and FLNA (Abnova, 1:1000, monoclonal antibody, #H00002316-M01) and then anti-mouse HRP- linked antibodies (Cell Signalling 1:2000, #7076) were used to detect the total levels of the SST2 and FLNA proteins. Densitometric data were normalized to vinculin antibody (#4650, Cell Signalling, 1:1000).

The 1:10000 dilution of anti-cyclin D1, (monoclonal antibody, 04-1151, EMD Millipore) and anti-rabbit HRP- linked antibodies (Cell Signalling, 1:2000, #7074) were used to detect cyclin D1 expression levels. Monoclonal anti-GAPDH (AM4300, Ambion, 1:4000) was used as housekeeping, developed using an anti-mouse secondary antibody.

To detect phosphorylated ERK1/2, 1:2000 dilution of antiphospho-p42/44 monoclonal antibody (#4370, Cell Signalling) and an anti-rabbit antibody were used. The presence of total ERK1/2 was analysed by stripping and

![Figure 1](http://erc.endocrinology-journals.org/)

**Figure 1**

FLNA expression in P-NETs. (A) Immunoblots of FLNA and SST2 performed on eight different neuroendocrine tumour samples. FLNA was expressed at variable levels in different samples, without correlation with SST2 expression. (B) Representative pictures of immunohistochemistry for FLNA and SST2 in different GEP-NETs (20× magnification). FLNA and SST2 pictures in the same column correspond to the same tumour. Confirming western-blot data, no correlation between SST2 and FLNA expression have been found.
reprobing with anti-total p42/44 monoclonal antibody (1:1000; Cell Signalling, #4695) and developed using an anti-rabbit secondary antibody. The 1:1000 dilution of VEGF (Abcam, ab46154, polyclonal antibody) and an anti-rabbit antibody were used. GAPDH was used as housekeeping.

After enzyme-linked chemiluminescence membrane incubation (Immobilon Western Chemiluminescent HRP Substrate, Millipore), chemiluminescence was detected using the ChemiDoc-MP Imaging System (Bio-Rad Laboratories, Inc.) and analysed using the image analysis program NIH ImageJ. Experiments were repeated three times.

**cAMP assay**

To quantify the inhibition of forskolin-induced cAMP accumulation, QGP1 cells transfected with FLNA siRNA or negative control siRNA were preincubated with 0.5 mM 3-isobutyl-1-methylxantine (IBMX) for 30 min, and subsequently with 1 mM forskolin with or without BIM23120 10 nM for 30 min at 37 °C. Intracellular cAMP was measured by enzymatic immunoassay (Promega).

**VEGF secretion study**

To analyse VEGF secretion, QGP1 cells were distributed in 24-well tissue culture plates and incubated with FLNA siRNA or negative control siRNA for 72 h. After incubation, cells were treated with or without BIM23120 10 nM in serum free RPMI-1640 medium for 72 h at 37 °C. Collected supernatants were used to measured VEGF concentration with ELISA kit (Invitrogen), according to manufacturer instructions. The absorbance was measured in a microplate reader at 450 nm and the sample values of VEGF were read off the standard procedure.

**Cell adhesion assay**

To analyse the role of FLNA in mediating SST2 effects on cell adhesion in QGP1 and P-NETs primary cultures, cells

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**Table 1** Pathological findings and FLNA/SST2 score immunoreactivity of P-NETs included in the IHC study

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FLNA and SST2 immunoreactivities were graded taking into account both the percentage of positive cells (0–30% = 1, 31–60% = 2, 61–100% = 3) and the staining intensity (0 = absence of immunoreactivity; 1 = weak; 2 = medium intensity; and 3 = strong reactivity) and evaluating at least 100 cells per section. G1 = low tumour grade, G2 = intermediate tumour grade, G3 = high-tumour grade. HPF = high power fields. Ki-67% = marker to quantify the proliferative rate.
with or without FLNA siRNA were plated onto a collagen type IV-coated 48-well plate and incubated with or without BIM23120 10 nM in serum-free medium for 90 min at 37 °C, as by manufacturer’s protocol (Cell Biolabs, Inc., San Diego, CA, USA). Briefly, nonadherent cells were removed by gently washing plates 4–5 times with PBS, adherent cells were lysed and subsequently detected with CyQuant GR Dye (Cell Biolabs, Inc.). Finally, each extracted sample was quantified by measuring fluorescence at 480 nm/520 nm. The experiment was performed four times in quadruplicate.

Cell migration assay
To analyse the role of FLNA in mediating SST2 effects on cell migration, QGP1 cells silenced or not were plated in polycarbonate membrane plate (Cell Biolabs, Inc.) with or without BIM23120 10 nM in serum-free RPMI and placed into the feeder tray, containing chemoattractant solution, according to the instruction of the manufacturer. After 24 h of incubation at 37 °C, migratory cells were first dissociated from the membrane, then stained and quantified after extraction using a fluorometric plate reader.

Statistical analysis
The results were expressed as mean ± s.d. A paired two-tailed Student’s t-test was used to detect the significance between two series of data. P < 0.05 was accepted as statistically significant.

Results
FLNA is not involved in SST2 expression in P-NETs
We first evaluated FLNA and SST2 expression in P-NETs by both western blotting (n = 8) and immunohistochemistry (n = 29). FLNA was expressed at variable levels in different samples (Fig. 1A and B), without significant correlation with SST2 expression. In addition, as shown in Table 1, we did not find any correlation between clinical phenotype of different P-NETs and immunohistochemistry score of both proteins.

FLNA is not required for SST2 expression in basal condition but it reduces SST2 expression after long-term agonist stimulation in QGP1 cells
To overcome the limitations due to the low availability of fresh neuroendocrine tumour samples and the low number of cells obtained, we used human pancreatic neuroendocrine cell line QGP1, endogenously expressing SST2 and FLNA, as a model for P-NET.

We analysed the effects of FLNA knockdown by siRNA against FLNA in QGP1 cells. Three days after transfection, in the absence of additional stimuli, western blot analysis showed a significant decrease of FLNA levels with respect to negative control, without affecting SST2 protein expression (Fig. 2A), these data suggesting that in neuroendocrine tumours FLNA is not required for SST2 expression in basal conditions. Thus, we decided to study the possible involvement of FLNA on SST2 down-regulation after agonist stimulation. To test the impact of FLNA on SST2 stability, QGP1 cells were transfected with C-siRNA and FLNA siRNA and then incubated with or without BIM23120 10 nM. As shown in Fig. 2B, long-term BIM23120 treatment (72 h) induced a significant reduction in SST2 expression in cells silenced for FLNA (−43 ± 21%, P < 0.05 vs untreated cells). Conversely,
in control cells BIM23120 treatment did not affect SST2 protein levels as evaluated by western blot.

**FLNA is required for SST2 signalling in QGP1 cells**

To investigate the role of FLNA in SST2 signalling, we evaluated the somatostatin analogue inhibition of forskolin-induced intracellular cAMP accumulation, that reflects the reduction in adenylyl cyclase activity in the presence of phosphodiesterase inhibitors. BIM23120 at 10 nM inhibited forskolin-stimulated cAMP accumulation in cells transfected with negative control siRNA (−24 ± 3%, \( P < 0.05 \) vs basal) (Fig. 3A), while this effect was abrogated in FLNA silenced cells.

To study the impact of FLNA in SST2-mediated inhibition of cell proliferation, we analysed the effects of SST2 activation on extracellular signal-regulated kinase (ERK1/2) phosphorylation, that mediates the anti-mitotic effect of SS analogue. BIM23120 significant decreased ERK1/2 phosphorylation in control cells (−42 ± 14% in negative control transfected cells, \( P < 0.05 \) vs untreated cells) and this reduction was totally abolished in silenced cells (Fig. 3B).

In addition, we further studied the antiproliferative effects of SST2 by evaluating the reduction of cyclin D1 levels induced by the specific SST2 agonist BIM23120 in FLNA-silenced QGP1 cells (Fig. 3C). As shown, the reduction of cyclin D1 levels observed in control cells after 6 h of BIM23120 incubation (−46 ± 18%, \( P < 0.05 \) vs untreated cells) was completely ablated after FLNA silencing.

**FLNA is involved in SST2-mediated angiogenesis inhibition**

To study the role of FLNA in SST2-mediated angiogenesis inhibition, we analysed the effects of SST2 on VEGF protein expression and secretion. As expected BIM23120 incubation induced a decrease in both VEGF expression (−31 ± 5% in negative control transfected cells, \( P < 0.01 \) vs untreated cells) and in vitro release (−40 ± 24% in negative control transfected cells, \( P < 0.05 \) vs untreated cells), these effects being completely abolished by FLNA silencing (Fig. 4A and B).

**FLNA is involved in SST2-mediated effects on cell adhesion and migration**

To analyse the involvement of FLNA in SST2-mediated cell adhesion, a pivotal event in the cancer development and progression, we examined the effects of SST2 on cell adhesion by incubation of BIM23120 in FLNA silenced P-NETs primary cultured cells. Interestingly, BIM23120 and abolished in FLNA siRNA transfected QGP1 cells. The graph shows the quantification of phospho-ERK1/2 normalized to total ERK1/2 (mean ± s.d. from three independent experiments). Values represent mean ± s.d. *\( P < 0.05 \) vs corresponding basal. (C) Effect of BIM23120 treatment (10 nM) on cyclin D1 expression on cells transfected for 72 h with C- siRNA or FLNA siRNA. In C- siRNA BIM23120 caused a significant inhibition of cyclin D1 levels, whereas no effect on cyclin D1 expression was mediated by SST2 in FLNA siRNA. Experiments were repeated at least three times. Values represent mean ± s.d. *\( P < 0.05 \) vs corresponding basal.
promoted cell adhesion in control cells (+46 ± 2%, P < 0.01 vs basal) and this effect was completely abolished in cells lacking FLNA (Fig. 5A).

To confirm the results obtained with P-NET cells, SST2-mediated stimulation of cell adhesion was tested in QGP1 cells transfected for 72 h with FLNA siRNA or negative control siRNA. As expected, QGP1 cells expressing FLNA showed an increase of cell adhesion by BIM23120 (+86 ± 45% at 10 nM, P < 0.05 vs basal), that was abrogated in FLNA silenced cells (Fig. 5B).

We then analysed the regulation of cell migration mediated by SST2. Exposure of QGP1 cells transfected with the negative control siRNA to SST2 selective agonist BIM23120 caused a reduction of cell migration at 10 nM (−24 ± 2%, P < 0.00001 vs basal), this inhibitory effect being completely lost in FLNA silenced cells (Fig. 5C).

Discussion

In the present study we demonstrated that FLNA, a widely expressed cytoskeleton protein, has a crucial role in SST2 stabilization at the membrane level, but it is not involved in SST2 expression. Contrary to what observed in PRL-secreting adenoma, in which low D2R expression is associated with low FLNA levels (Peverelli et al. 2012), and in agreement with what demonstrated in GH-secreting adenoma (Peverelli et al. 2014), no correspondence between FLNA and SST2 expression was found in P-NETs. Moreover, no significant correlations were found between FLNA expression and either clinical presentation or pathological findings (i.e. grading), this finding being in contrast with other cancer types. In fact, it has been
demonstrated that FLNA is overexpressed in multiple types of cancer, including prostate, breast, lung cancer, hemangiomias, colon cancer, melanoma, neuroblastoma, squamous cell carcinoma, hepatic cholangiocarcinoma, this overexpression possibly correlating with cancer aggressiveness (Savoy & Ghosh 2013, Yue et al. 2013). To confirm the result obtained by immunohistochemistry and western blot in a series of human P-NETs, we performed further experiments on QGP1 cells, an appropriate cellular model that expresses endogenously FLNA and SST2. We showed that FLNA silencing did not result in receptor levels modifications in QGP1 cells, thus suggesting that SST2 expression is not dependent on FLNA levels. Although our data demonstrated that FLNA is not required for SST2 basal expression, we then investigated the possible role of FLNA on SST2 stabilization after prolonged agonist stimulation in QGP1 cells. Our results showed that in QGP1 cells FLNA knockdown reduced SST2 stability after long-term agonist incubation, whereas BIM23120 did not affect SST2 protein levels in control cells. Our observation is in agreement with recent data carried out on GH-secreting adenoma (Peverelli et al. 2014) and on FLNA deficient melanoma cell lines M2 (Najib et al. 2012). Although, further studies will be needed to fully understand how FLNA regulates SST2 stability, several evidences indicate that FLNA plays a crucial role in trafficking and stabilizing several GPCRs on plasma membrane (Liu et al. 1997, Seck et al. 2003, Thelin et al. 2007). In this respect, it is worth noting that FLNA directly interacts with β-arrestins (Scott et al. 2006), suggesting that a complex receptor–FLNA–arrestin may be possibly involved in SST2 down-regulation processes.

In neuroendocrine tumours, the modification of receptor stability resulting from FLNA silencing has a profound impact on SST2 signalling. In fact, in the absence of FLNA the selective SST2 agonist was unable to trigger the most relevant biological responses mediated by SS analogues (i.e. inhibition of cell proliferation). In particular, our data demonstrated that FLNA knockdown resulted in the loss of the ability of SST2 agonist to reduce intracellular cAMP accumulation, ERK 1/2 phosphorylation and cyclin D1 levels.

Moreover, we demonstrated that FLNA is required for mediating SST2 inhibitory effect on angiogenesis that represents a crucial cellular event for cancer progression. In particular, it has been demonstrated that FLNA physically interacts with HIF-1α (Zhenga et al. 2014), that regulates angiogenesis through upregulation of vascular endothelial growth factor (VEGF) (Uramoto et al. 2010, Berardi et al. 2011, Semenza 2012). Whereas VEGF-driven angiogenesis may play an important role in endocrine tumourigenesis and tumour progression (Hanahan et al. 1996) and several in vitro studies suggested that SS analogues display potent antiangiogenic properties (Woltering et al. 1991, Barrie et al. 1993, Danesi & Del Tacca 1996, Kumar et al. 2004), we showed that SST2 agonist reduced VEGF protein levels and release, but this inhibitory effect was totally abolished in the absence of FLNA.

In addition, considering that pancreatic neuroendocrine tumours are characterized by widely disseminated metastatic disease at diagnosis, we focused on two key events in tumour development, such as cell adhesion and migration. In particular, to the best of our knowledge, our study is the first to characterize SST2 selective agonist effect on cell adhesion and migration in neuroendocrine tumours. According to the few studies on the role of somatostatin analogues on cell motility that have been carried out on other cellular system (Talme et al. 2001, Tang et al. 2010), we demonstrated that somatostatin analogue promoted cell adhesion and inhibited cell migration. To study the implication of FLNA on SST2 regulation of cancer development, we modulated FLNA expression, providing evidence that FLNA silencing abolished stimulatory and inhibitory effect of SST2 agonist on cell adhesion and cell migration respectively. These data suggested that FLNA is crucial for regulation of SST2-mediated cell mobility in pancreatic neuroendocrine tumour cells.

In conclusion, these results provide new evidence for a role of cytoskeleton in the control of neuroendocrine tumour progression triggered by SST2. In particular, our data demonstrated that FLNA is required to maintain SST2 stability after agonist stimulation, to mediate SST2 signal transduction and to regulate angiogenesis and cell motility.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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