Internalization-defective mutants of somatostatin receptor subtype 2 exert normal signaling functions in hematopoietic cells

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Received 16 July 2001; accepted 19 July 2001

First published online 1 August 2001

Edited by Felix Wieland

Abstract The regulatory peptide somatostatin (SST) acts via a family of G-protein-coupled receptors comprising five subtypes (SSTR1–5). G-protein-coupled receptors activate multiple signaling mechanisms, which variably depend on internalization and intracellular routing of activated receptors. We have recently demonstrated that hematopoietic precursors express SSTR2 and that SST is a chemoattractant for these cells. Herein, we characterize critical regions in SSTR2 involved in endocytosis and describe how ligand-induced internalization impacts on two major signaling functions of SSTR2 in hematopoietic cells, the activation of the Erk pathway and the induction of promigratory responses.

Key words: Somatostatin; Receptor subtype; Internalization; Hematopoietic cell; Chemoattractant

1. Introduction

The somatostatin (SST) peptides SST-14 and SST-28 regulate a variety of cellular functions through binding to a family of G-protein-coupled receptors (GPCRs) [1]. Five subtypes of human somatostatin receptors (SSTRs) have been identified, which are encoded by related genes localized on different chromosomes [2]. Although individual SSTR subtypes share a high degree of structural conservation and display some overlapping functions, they also exhibit distinct functional properties, for example with regard to ligand binding and kinetics of receptor internalization [3–5]. A common property of GPCRs is their ability to regulate responsiveness to continued agonist exposure [6]. Typically, this involves desensitization of the receptor due to uncoupling from G-proteins, as well as receptor internalization and degradation [6]. Internalization and intracellular trafficking of GPCRs may have a major impact on the signaling properties of these receptors. For instance, it was demonstrated that Erk1/Erk2 activation by lysophosphatidic acid, thrombin and β2 adrenergic receptors is endocytosis-dependent [7].

The structural determinants involved in the internalization of receptor subtypes SSTR1–5 have been partly characterized. Mutational analysis has demonstrated that phosphorylation of cytoplasmic threonine and serine residues in the carboxyl terminus of SSTR2 plays a major role in agonist-induced desensitization and internalization [3,8,9]. Notably, internalization of SSTR subtypes may vary considerably depending on the cell type studied [3,4].

We have recently shown that SSTR2 is expressed in both human and mouse hematopoietic systems and that SST acts as a potent chemoattractant for hematopoietic cells. Interestingly, SSTR2 expression is restricted to primitive ($CD34^+$) precursor cells [10]. SSTRs are also expressed in various hematological malignancies, including lymphomas [11,12], acute myeloid leukemias (AML) and lymphoblastic leukemias [13–15]. Similar to normal hematopoietic progenitors, human AML cells express SSTR2, but none of the other SSTR subtypes [15]. Whether ligand-induced internalization of SSTR2 takes place in hematopoietic cells and how this influences SST-induced responses of these cell types has not been established. In the present study, we show that the majority of SSTR2s are rapidly internalized upon ligand binding and identify a dileucine-containing motif in the C-terminus of SSTR2 as being an important regulatory domain for endocytosis. Quite surprisingly, we observed that two major signaling functions of SSTR2 in hematopoietic cell types, activation of the Erk pathway and induction of chemotaxis, are not significantly altered when internalization of SSTR2 is prevented by truncating the C-terminal region of the receptor.

2. Materials and methods

2.1. Plasmid construction

Human SSTR2 cDNA (a kind gift from Dr G.I. Bell, Howard Hughes Medical Institute, Chicago, IL, USA) was excised from the pBluescript vector and inserted into the *Bam*HI/*Sal*I site of the eukaryotic expression vector pBABE [16]. The LL→AA mutant (SSTR2-L360/361A) and the deletion mutant (SSTR2-Δ342) were constructed by recombinant PCR, using the following oligonucleotide primers: SH2/592-F (5′-TCTGCTGGTCATCTTGCCCATCAT), SH2/1153LL-F (5′-AGGACCGCGGCTAATGGAGACCTCCAAAC), SH2/1172LL-R (5′-TCCATTAGCCGCGGTCCTCTGGGTCTCCG), T3-R (5′-GCGCAATTAACCCTCACTAAAGGG), Δ342-F (5′-GGAGTGACTGAAAGCAGGACAAATCC) and Δ342-R (5′-TCCTGCTTTCAGTCACTCCGCTTCCCC). The 5′ segment of mutant SSTR2-L360/361A was amplified using SH2/592-F and SH2/1172LL-R, and the 3′ segment using SH2/1153LL-F and T3-R. The 5′ segment of mutant SSTR2-Δ342 was amplified using SH2/592-F and Δ342-R, and the 3′ segment using Δ342-F and T3-R. Products

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Abbreviations: GPCR, G-protein-coupled receptor; GRK, G-protein receptor kinase; PKA, protein kinase A; PKC, protein kinase C; SST, somatostatin; SSTR, somatostatin receptor; wt, wild-type

0014-5793/01/$20.00
PII: S0014-5793(01)02729-6

of the primary PCRs were isolated, mixed 1:1, and used as a template for a secondary PCR with SH2/592-F and T3-R. The products were digested with *Bst*EII and *Sal*I and cloned into pBABE containing wild-type (wt)-SSTR2, which had also been digested with these enzymes. The authenticity of all mutants was verified by restriction-enzyme analysis and DNA sequencing.

2.2. Cells and transfections

The IL-3-dependent murine myeloid cell line 32Dcl10 [17] was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng/ml murine IL-3 at 37°C and 5% CO_2. To obtain stable transfectants, cells were electroporated with 10 μg *Pvu*I-digested pBABE, pBABE[wt-SSTR2], pBABE[SSTR2-L360/361A] and pBABE[SSTR2-Δ342], using a progenitor apparatus set at 230 V, 100 μF, and 1 s. After 48 h of incubation, cells were selected with puromycin at a concentration of 1 μg/ml. Multiple clones were expanded for further analysis.

2.3. Flow cytometry

To determine SSTR2 expression levels, cells were incubated with 50 nM of Fluo-somatostatin™ (Fluo-SST; Advanced Bioconcept, Montreal, QC, Canada) for 45 min at room temperature in the dark with non-specific binding assessed by including 100-fold excess of (D-Trp^8)-SST-14 (BACHEM AG, Switzerland). Cells were subjected to flow-cytometric analysis on a FACScan (Becton-Dickinson, Sunnyvale, CA, USA).

2.4. Internalization experiments

The SST analog [Tyr^3]octreotide (Novartis Pharma, Basel, Switzerland) was iodinated with ^{125}I by chloramine-T method and purified by high-performance liquid chromatography, as described previously in detail [18]. The specific radioactivity of the radioligand was approximately 2000 Ci/mmol. Internalization of the radioligand was assessed as described previously [19]. In brief, 32D[wt-SSTR2] cells were incubated with approximately 200 000 cpm [^{125}I–Tyr^3]octreotide (0.1 nM final concentration) at 37°C for the times indicated. Excess unlabeled octreotide (1 μM; Novartis, Basel, Switzerland) was added in control incubations to determine non-specific membrane binding and internalization. After incubation, surface bound radioligand was removed with 1 ml acid wash (20 mM sodium acetate, pH 5.0) for 10 min [20]. Internalized radioligand was measured as acid-resistant counts in 0.1 N NaOH extracts of acid washed cells [20].

Internalization was also analyzed by flow cytometry. For this purpose, cells were incubated at 37°C with octreotide in internalization medium (RPMI 1640+0.25% bovine serum albumin (BSA)). To terminate the incubation, cells were washed twice with ice-cold internalization medium. Cell surface-bound ligand was removed by resuspending the cells in 1 ml sodium acetate (20 mM) in Hanks' balanced solution, pH 5.0 (HBSS-Ac), and incubating for 10 min at room temperature. Subsequently, cells were washed with PS (PBS+1% FCS) to remove the HBSS-Ac, stained with Fluo-SST and subjected to flow-cytometric analysis on a FACScan (Becton-Dickinson, Sunnyvale, CA, USA) as described above.

2.5. Cell lysates and Western blotting

Preparation of cell lysates and Western blotting was performed as described [21]. Cells were stimulated with 10^{-6} M octreotide in RPMI 1640, with 100 ng/ml granulocyte-colony stimulating factor (positive control) or with RPMI 1640 alone. Antibodies used for Western blotting were α-Erk1 and α-phospho-Erk (Tyr204) (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA).

2.6. Migration assay

Migration was assessed in transwell culture dishes with 5 μm pore filters (Transwell, 6.5 mm diameter, 24-well cell clusters; Costar, Cambridge, MA, USA). Cells ($1–2\times10^5$) suspended in 100 μl of migration buffer (Iscove's medium, 0.5% BSA) were placed in the upper chamber. Migration buffer (0.6 ml) containing increasing concentrations of octreotide was placed in the lower chamber of the transwell system. Chambers were maintained at 37°C, 5% CO_2, for 4 h. Cells that had migrated into the lower chamber were counted using a cell counter (CASY®1/TTC, Schärfe Systems, Germany).

3. Results and discussion

3.1. Expression of SSTR2 and SSTR2 mutants on myeloid 32D cells

The structural features of two mutants of SSTR2, SSTR2-

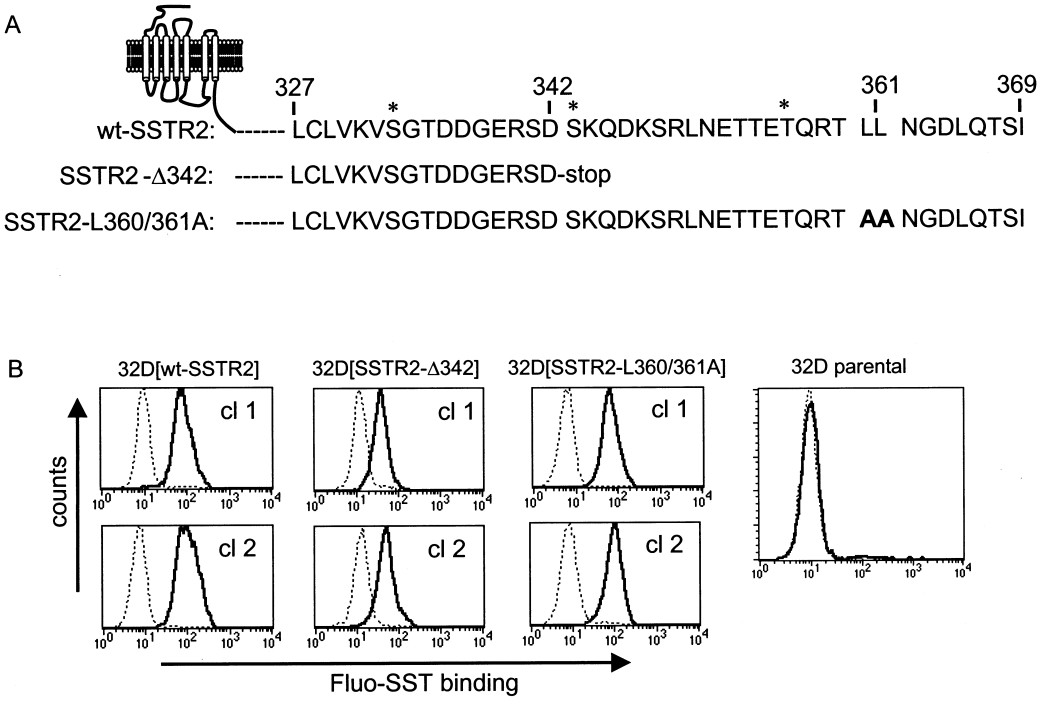

Fig. 1. Expression of SSTR2 constructs in 32D cells. A: The carboxyl-terminal sequences of SSTR2 and position of mutations. Asterisks indicate potential PKA/PKC phosphorylation sites. B: Flow-cytometric analysis of receptor expression on representative clones of 32D[wt-SSTR2], 32D[ΔSSTR2], 32D[SSTR2-L360/361A] and 32D parental. Amount of fluorescence after labeling with Fluo-SST is shown by the solid lines. The broken lines represent the fluorescence that remains after displacement of Fluo-SST by 100-fold molar excess of non-labeled SST.

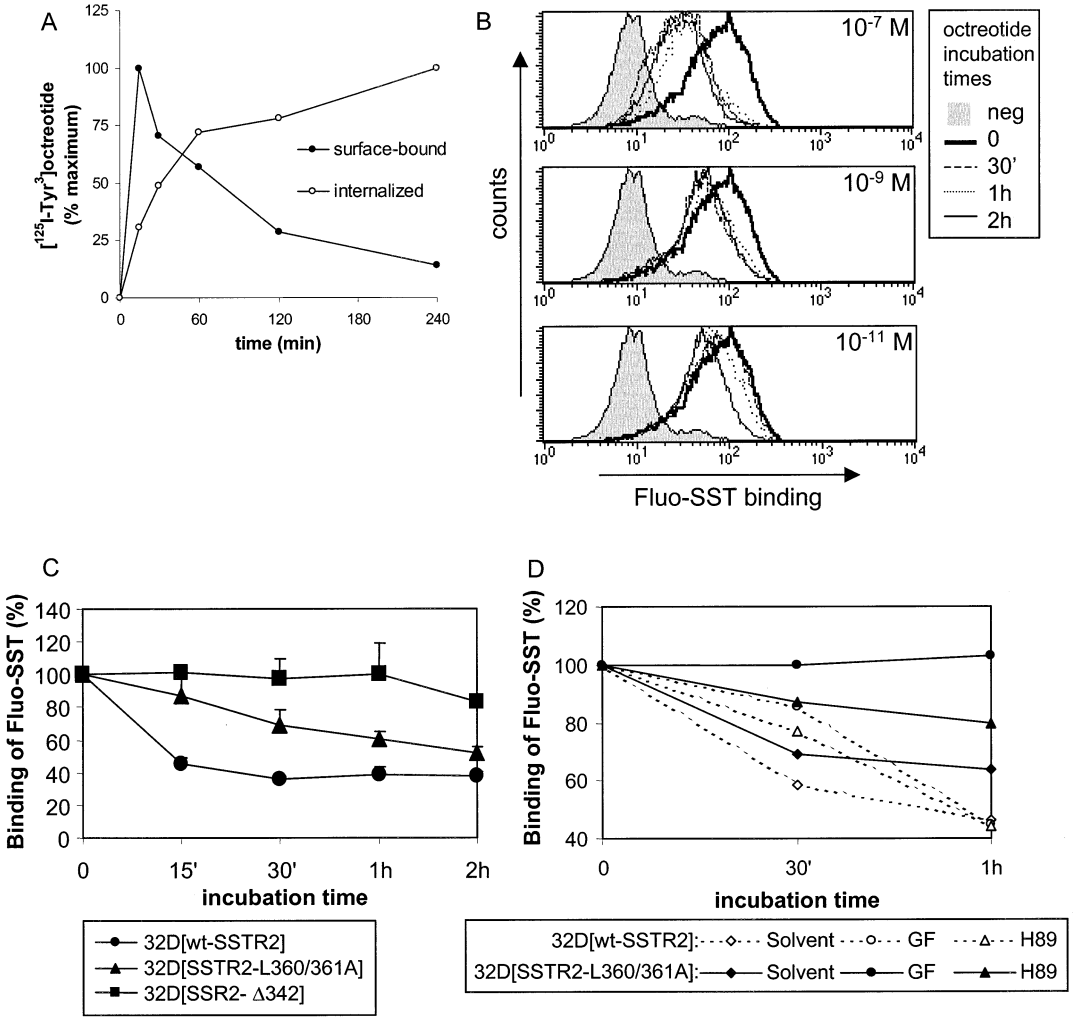

Fig. 2. Internalization of SSTR2 mutants. A: Time course of membrane binding and internalization of [^{125}I–Tyr^3]octreotide by 32D[wt-SSTR2] cells. Values are expressed as percent of maximum specific binding and internalization (maximum surface-bound: 2.3%; maximum internalization: 78%). Representative graph is shown of three independent experiments. B: FACS analysis of octreotide-induced internalization of SSTR2. 32D[wt-SSTR2] cells were incubated with octreotide for 30 min, 1 h and 2 h at 37°C at the concentrations indicated. After washing with acid to remove unbound and surface-bound ligand, cells were stained with Fluo-SST or with Fluo-SST and 100-fold molar excess of non-labeled SST to determine non-specific binding (shaded histograms). C: Comparative FACS analysis as performed under (B) on 32D[wt-SSTR2], 32D[SSTR2-Δ342] and 32D[SSTR2-L360/361A] with 10^{-9} M of octreotide. Results are expressed as percentages of specific Fluo-SST-binding at time zero. D: Effect of PKC inhibitor GF109203X and PKA inhibitor H89 on internalization of wt-SSTR2 and SSTR2-L360/361A. Cells were preincubated with the inhibitors for 30 min and 1 h at 37°C and further treated as under (C). A representative graph of three independent experiments is shown.

Δ342 and SSTR2-L360/361A are shown in Fig. 1A. Upon transfection in 32D cells, membrane expression of SSTR2 wt and mutants was determined by FACS analysis. Cell clones expressing equivalent levels of receptor proteins were selected for further analysis (Fig. 1B). Parental, as well as empty-plasmid controls, did not express endogenous SSTR, as confirmed by RT-PCR (data not shown) and FACS analyses (Fig. 1B).

3.2. Ligand-induced internalization of SSTR2 in hematopoietic cells

Previously, it was shown that SSTR3, SSTR4 and SSTR5 are internalized upon ligand binding and that internalization requires an intact C-terminal region of the receptor proteins [5,8,9,22,23]. In contrast, SSTR1 did not internalize upon continued ligand treatment [4,24]. We determined kinetics of binding and internalization of [^{125}I–Tyr^3]octreotide by 32D[wt-SSTR2] cells (Fig. 2A). The membrane bound fraction of radiolabeled octreotide increased rapidly during the first 30 min and then gradually declined to approximately 25% of maximal values after 240 min of incubation. Internalization of radioligand reached a plateau at 60 min with approximately 75% of the dose of radioligand internalized.

3.3. Internalization and signaling function of SSTR2 mutants

The kinetics and magnitude of internalization of SSTR2 and mutants with different concentrations of ligand were determined by flow cytometry using Fluo-SST. Based on the results from the radioreceptor assay, the analysis was performed 15 min, 30 min, 60 min and 120 min after initiation of incubation with octreotide. An example of this analysis on 32D[wt-SSTR2] is shown in Fig. 2B. Subsequently, the internalization kinetics of wt-SSTR2 and SSTR2-Δ342 were compared. Whereas 60% of wt-SSTR2 was internalized upon a 15-min incubation with 10^{-9} M of octreotide, cell surface expres-

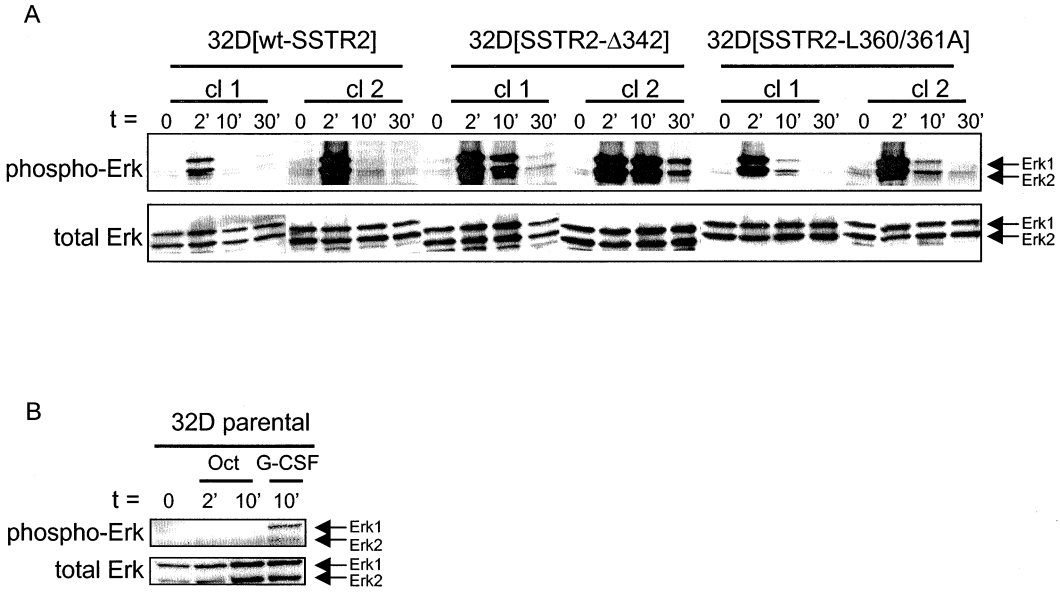

Fig. 3. Octreotide-induced phosphorylation of Erk1 and Erk2 in (A) 32D[wt-SSTR2], 32D[SSTR2-Δ342], 32D[SSTR2-L360/361A] and (B) 32D parental cells. Cells were treated with 10^{-6} M octreotide (Oct) at 37°C for the times indicated. As a positive control, stimulation with 100 ng/ml granulocyte-colony stimulating factor (G-CSF) was included. Western blotting was performed using antibodies against phospho-Erk (Tyr204). To control for equal loading, blots were stripped and restained with antibodies against total Erk.

sion of SSTR2-Δ342 was hardly affected by ligand binding (Fig. 2C). These results confirm that the C-terminal part of SSTR2 contains motifs that are crucial for internalization [8,23,25]. However, it cannot be excluded that these sequences are also involved in the control of recycling of the receptor to the membrane.

Serine and threonine residues in the C-terminus of SSTR are phosphorylated by different serine/threonine kinases and have been implicated in agonist-induced desensitization and internalization of GPCR's [8]. These kinases include the second-messenger kinases protein kinase A (PKA) and protein kinase C (PKC). However, both PKA and PKC inhibitors H89 and GF109203X only partly reduced octreotide-induced internalization of wt-SSTR2 (Fig. 2D).

The C-terminus of SSTR2 also comprises a dileucine motif, L360L361 in the context TQRTLL, that could potentially function as a binding site for adapter proteins involved in internalization and intracellular protein trafficking [26,27]. Mutation of this motif (L360/361A) resulted in a significant reduction of ligand-induced internalization (Fig. 2C). Interestingly, internalization of SSTR2-L360/361A could be completely blocked by GF109203X and partially by H89, indicating that mechanisms linked to the dileucine motif and PKA or PKC-mediated phosphorylation cooperate in the internalization of SSTR2 (Fig. 2D). Dileucine motifs are also present in the C-tail of SSTR3 (FRRVLL and SKEQQLL) and SSTR4 (LRCCLL), but their role in internalization remains to be established.

GPCRs are also phosphorylated by members of the G-protein receptor kinase (GRK) family, which results in binding of the receptors to β-arrestin [6,28,29]. Recent work on the β-adrenergic receptor has suggested that β-arrestin functions as an adapter molecule that connects the receptor to the clathrin-mediated endocytosis machinery and to the cytoplasmic tyrosine kinase c-Src. Interaction of the β-adrenergic receptor with clathrin is required for the activation of Erks [7]. We found that inhibition of GRK activity by using the non-selective GRK inhibitors, Zn^{2+} and heparin [30], did not affect SST-induced receptor internalization and Erk phosphorylation in 32D[SSTR2] cells (data not shown). Rather, Erk phosphorylation was sustained after activation of the internalization-defective SSTR2 mutants (Fig. 3A). In addition, the Src inhibitor PP1 had no effect on SSTR2-mediated Erk activation (data not shown). Thus, unlike β-adrenergic receptor, GRK-mediated internalization and c-Src activity are not critical for SSTR2-induced Erk activation in hematopoietic cells. As expected, no octreotide-induced activation was seen in 32D parental cells that lack expression of SSTR (Fig. 3B).

3.4. SST-induced migration of hematopoietic cells is not influenced by SSTR2 internalization

SST exerts multiple effects on cells of the neuroendocrine and immune systems, mainly as an inhibitor of secretory and proliferative responses. We have recently identified a novel function of SST in the hematopoietic system, where it acts as a potent chemoattractant for normal and leukemic primitive progenitor cells expressing SSTR2 [10,15]. The migratory responses of hematopoietic cells to SST showed a typical bell-

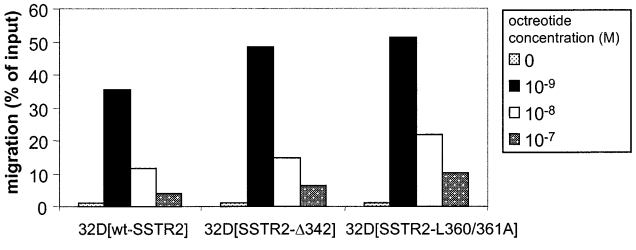

Fig. 4. Migration of 32D[wt-SSTR2], 32D[SSTR2-Δ342] and 32D[SSTR2-L360/361A] cells in response to increasing concentrations of octreotide. A representative graph of three experiments is shown.

shaped dose-response relationship, with maximal effects seen in the nanomolar range. One explanation for the loss of response at higher ligand concentrations is that surface expression of SSTR2 rapidly becomes insufficient under these conditions, due to increased and sustained internalization and proteasomal degradation of the receptor proteins. We tested this possibility by comparing the migration properties of 32D[wt-SSTR2] with 32D cells expressing the internalization-defective mutants SSTR2-Δ342 and SSTR2-L360/361A. Strikingly, we found that the inhibition of migration at concentrations of 10^{-8} M and 10^{-7} M of octreotide was not alleviated by the mutations in the SSTR2 C-terminus (Fig. 4), indicating that changing the internalization kinetics of the receptor does not alter the biphasic responses of hematopoietic cells to SST. It thus seems most likely that SSTR2 desensitization, rather than internalization and degradation, is the major mechanism by which hematopoietic cells downmodulate their responses to SST.

Acknowledgements: This work was supported by the Netherlands Organization for Scientific Research (NWO). We thank Dr. Marieke von Lindern for critical reading of the manuscript.

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