SOMATOSTATIN RECEPTORS IN THE HEMATOPOIETIC SYSTEM

Printed by: Optima Grafische Communicatie, Rotterdam, The Netherlands

Cover by Egied Simons, Karola van Rooyen en Sigrid Oomen

ISBN 90-77017-40-2

SOMATOSTATIN RECEPTORS IN THE HEMATOPOIETIC SYSTEM

SOMATOSTATINE RECEPTOREN IN HET HEMATOPOÏETISCH SYSTEEM

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof. dr. ir. J.H.van Bemmel
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden
op woensdag 23 januari 2002 om 9:45 uur

door

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Geboren te Oosterhout (NBr)

PROMOTIE COMMISSIE

Promotoren:	Prof. dr. I.P. Touw Prof. dr. B. Löwenberg
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The work described in this thesis was performed at the Institute of Hematology, Erasmus University Rotterdam, The Netherlands.
This work was supported by a grant from the Netherlands Organization for Scientific Research (NWO).

Aan mijn ouders

CONTENTS

Chapter 1

INTRODUCTION

partly published in

Somatostatin receptors in the haematopoietic system
Oomen SP, Hofland LJ, van Hagen PM, Lamberts SW, Touw IP

European Journal of Endocrinology (2000) **143** S9-S14

1.1 Scope of the thesis

Multiple interactions exist between the immune, hematopoietic, endocrine and nervous systems [1,2]. The bi-directional communication between the immune/hematopoietic and nervous systems is mediated by complex mechanisms involving multiple soluble factors (e.g., neuropeptides, neurotrophic factors, neurotransmitters and cytokines) produced by each system [3-5]. Examples of such factors are the neurotransmitter neuropeptide Y [6], produced by megakaryocytes [7], substance P (SP), which enhances the proliferation of primitive bone marrow cells and progenitors [4] and nerve growth factor (NGF), which contributes to differentiation of human basophils [8] and stimulates the release of inflammatory mediators from these cells [9]. A number of studies have demonstrated the expression of somatostatin receptors on cells derived from several hematological malignancies and have shown that somatostatin inhibits proliferation of these cells [10]. However, little is known of the effects of somatostatin on normal blood cell formation (hematopoiesis). This thesis comprises studies dealing with the role of somatostatin, a neuropeptide with multiple functions in the body, in hematopoiesis.

1.2 Hematopoiesis

During mammalian embryogenesis, the hematopoietic system is formed from mesodermally derived cells localized in the aorta-gonad-mesonephros (AGM) region and the yolk sac. At a later stage of development, fetal hematopoiesis takes place in the liver and subsequently shifts to the spleen and the bone marrow. The spleen then gradually becomes a less important hematopoietic organ and at birth hematopoiesis in humans is almost exclusively situated in the bone marrow [11].

Hematopoiesis is a strictly regulated process. All blood cells are derived from a small population of pluripotent stem cells that are capable of self-renewal and differentiation towards distinct lineage-committed progenitor cells. These committed progenitor cells can undergo proliferation, followed by terminal differentiation into the different mature blood cell types. Blood cells have a finite lifespan and must be replaced constantly throughout life. In addition to this requirement to maintain circulating cell numbers, it is also necessary to respond to host challenges with appropriately increased output of the specific cell types required. Finally, it is necessary to down-regulate output when the response is no longer required. This continuous production is tightly balanced and regulated by multiple mechanisms. Stromal cells and extracellular matrix in the bone marrow provide a suitable microenvironment required for hematopoietic cell development. In addition, a network of cytokines and hematopoietic growth factors (HGF) specifically controls the proliferation, differentiation, survival, and function of different hematopoietic cells. This network is particularly important under stress conditions, such as infection or bleeding, when rapid rises in specific blood cell types are needed.

Migration and homing of cells
As outlined above, during fetal development, the hematopoietic stem cells migrate from the fetal liver to the bone marrow (BM). This phenomenon of targeted migration via the circulation to a specific tissue is referred to as "homing" [12]. Regulation of progenitor cell mobilization and homing is a complex process, involving adhesion molecules, paracrine cytokines, and chemokines (Fig. 1). Two types of migration can be distinguished.

Chemoattraction is the unidirectional movement of cells towards a positive gradient of a compound, while chemokinesis reflects activation of cell

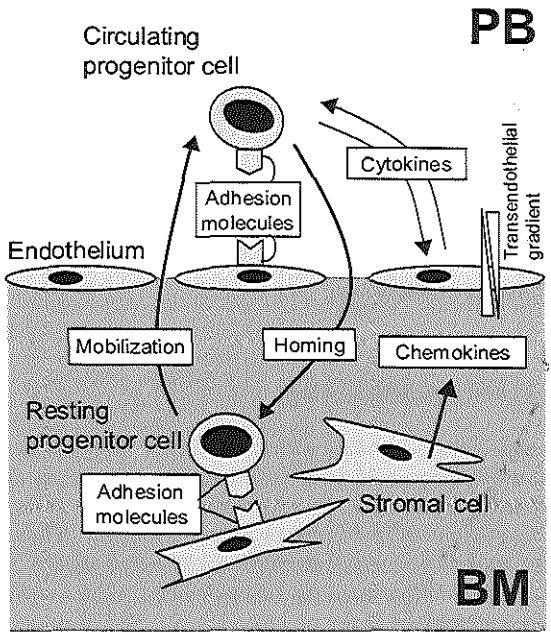

Figure 1. Mobilization and homing of hematopoietic progenitor cells are multifactorial processes that involve interactions via adhesion molecules, chemokines, and paracrine cytokines. Transendothelial migration most likely plays a role in hematopoietic progenitor cell trafficking. Adhesion molecules expressed on progenitor and bone marrow endothelial cells may regulate transition from resting to the circulating progenitor cell compartment and *visa versa*. Chemokines produced in the bone marrow stroma build up transendothelial gradients that may either support or inhibit migration of progenitor cells across the endothelial layer. In addition, endothelial cells can produce cytokines that influence proliferation and motility of progenitors, and hematopoietic progenitor cells may also produce cytokines that act on endothelial cells. PB: peripheral blood, BM: bone marrow. Redrafted with permission from [13].

motility and an induction of cell migration in a random direction. Chemokines comprise a large number of structurally related proteins that regulate migration and activation of leukocytes through G protein-coupled cell-surface receptors [14]. The first chemoattractant reported for human $CD34^+$ progenitor cells is stromal cell-derived factor-1 (SDF-1) [15]. This chemokine, produced by bone marrow stromal cells, is a ligand for the chemokine receptor CXCR4. *In vitro*, SDF-1 elicits maximal transendothelial migration of ~ 25% of the $CD34^+$ population 3h after exposure. SDF-1 is also a chemoattractant for human lymphocytes and monocytes [16]. Furthermore, SDF-1 and its receptor CXCR4 were found to be critical for murine bone marrow engraftment by human severe combined immunodeficient (SCID) repopulating stem cells [17]. Besides SDF-1, stem cell factor (SCF) has been shown to elicit some chemotaxis/chemokinesis on mouse progenitor cells. [18]. However, the percentage of migrating cells in response to SCF was lower, and maximal migration occurred much later (8-

24h) than seen in response to SDF-1 (2-4h), suggesting that SCF-induced migration might be due to indirect effects.
Migration of cells can be measured in an *in vitro* transwell migration assay [13]. Cells are placed in the upper chamber of the transwell and chemoattractant is placed in the lower chamber. The upper and lower chambers are separated by a microporous transwell membrane. After incubation transmigrated cells can be recovered from the lower chamber and quantified (Fig. 2).

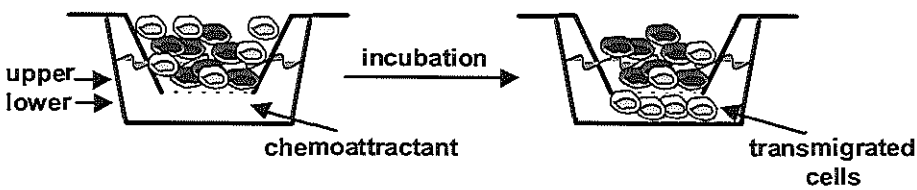

Figure 2. An *in vitro* transwell assay for cell migration.

1.3 Somatostatin (SST) and synthetic analogs

Somatostatin is a neuropeptide that is widely distributed in the body. It is secreted in two biologically active forms: a 14-amino acid form (somatostatin-14) and an amino-terminally extended 28-amino acid form (somatostatin-28). Like other peptide hormones, somatostatin is synthesized as part of a large precursor molecule (preprosomatostatin) that is rapidly cleaved into the prohormone form (prosomatostatin) and processed enzymatically to yield several mature products [19]. A disulfide bridge between two cysteine residues stabilizes the β-turn that forms the receptor-binding domain of the peptide (Fig. 2). SST was first identified as a growth hormone release-inhibiting factor synthesized in the hypothalamus [20]. Later, it was found that somatostatin is produced not only in the hypothalamus but throughout the central nervous system (CNS), where it acts as a neurotransmitter or paracrine/autocrine regulator and in most peripheral organs [19,21,22]. In peripheral tissues, SST regulates such diverse physiological processes as cell secretion, neuromodulation, smooth muscle contractility in the gastrointestinal tract, nutrient absorption or cell growth [19]. Somatostatin that is produced in the hypothalamus also travels through the portal circulation to the anterior pituitary, where it inhibits the secretion of growth hormone (GH) but also of other pituitary hormones such as thyroid-stimulating hormone (TSH) and prolactin (PRL). Finally, somatostatin is produced in specialized cells (D cells) in the gastrointestinal tract and the pancreas [23,24]. The major actions of SST are summarized in Table 1.

Since the discovery of somatostatin-14, its potent biological effects have generated a great deal of pharmacological and pharmaceutical interest in the development of potent compounds with selective actions for investigational and clinical use. Native somatostatin has a half-life in the circulation of less than 3 minutes. Hence, for effective pharmacotherapy with somatostatin, it was necessary to design analogs with greater metabolic stability than the naturally occurring peptides. Structure-activity studies of somatostatin-14 have shown that amino acid residues Phe^7, Trp^8, Lys^9, and Thr^{10}, which comprise a β-turn, are important for biological activity, with residues Trp^8 and Lys^9 being essential, whereas Phe^7 and Thr^{10} can undergo minor substitutions [25].

Localization	Action
CNS and PNS	Neurotransmitter
Pituitary gland	GH ↓, TSH ↓, PRL ↓
Salivary glands	Saliva production ↓
Parathyroid	Calcitonin ↓
Kidney	Renin ↓
Stomach	Acid secretion ↓, Pepsin ↓
Pancreas	Insulin ↓, Glucagon ↓, Enzyme secretion ↓
Gut	Intestinal ↓, Hormones ↓, Motility ↓

Table 1. Actions of somatostatin in different organ systems.
Derived from reference: [19]
CNS: central nervous system; PNS: peripheral nervous system; GH: growth hormone; TSH: thyroid-stimulating hormone; PRL: prolactin.

Many of the synthetic analogs are shortened versions of native somatostatin, keeping the disulfide-bridged loop with the receptor binding domain intact but modifying the N-terminal and sometimes C-terminal amino acid residues to make them more resistant to proteolytic degradation. The octapeptide analogs SMS201-995 (Octreotide), BIM23014 (Lancreotide) and RC160 (Vapreotide) are used clinically. The smallest somatostatin analogs are the hexapeptides, e.g. MK678 (Seglitide) [25] (Fig. 3).

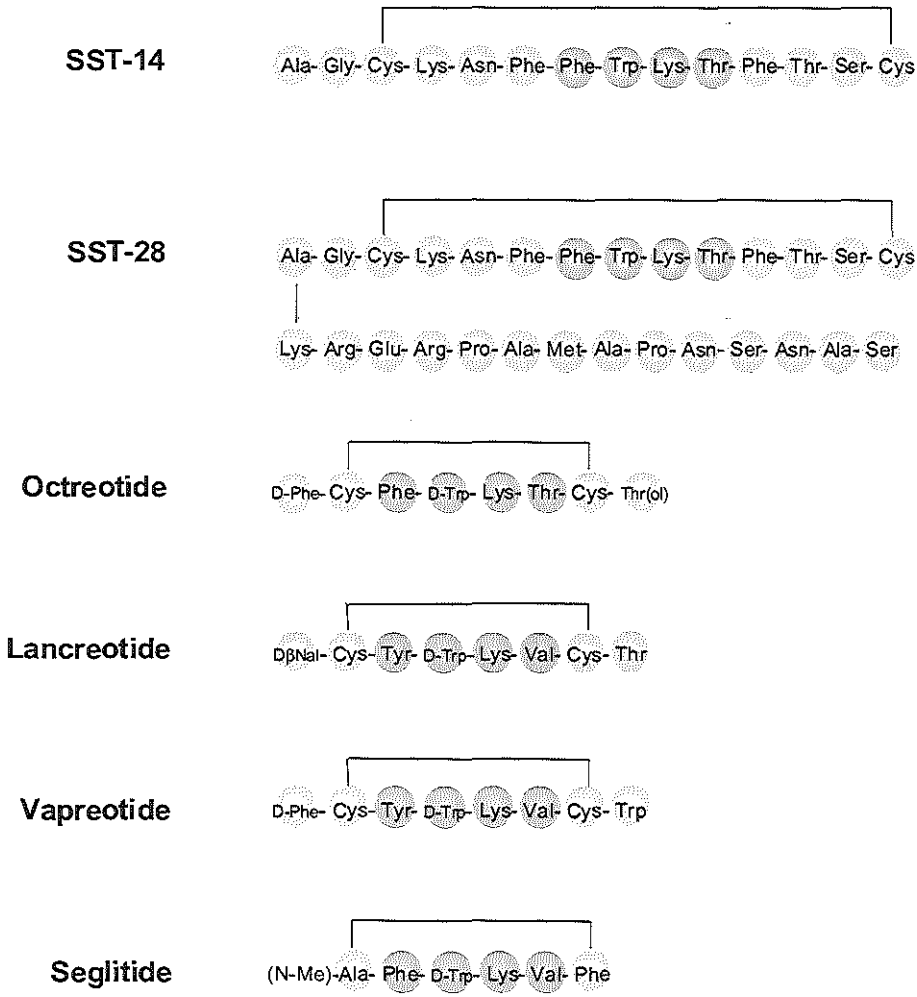

Figure 3. Amino acid sequences of native somatostatin (SST-14 and SST-28) and synthetic, metabolically stable somatostatin analogs used in the clinic.

1.4 Somatostatin receptors (SSTR)

Somatostatin acts through high-affinity plasma membrane receptors, which were first described in the pituitary GH_4C_1 cell line by whole-cell binding analysis [26].
Structurally, SSTR are so-called seven transmembrane domain (TMD) glycoproteins, comprising 7 membrane spanning α-helical domains connected by short loops, an N-terminal extracellular domain and a C-terminal intracellular domain. Hydrophobic and charged amino acids within the transmembrane domains 3, 6 and 7 are important for the interaction with the ligand [27,28]. However, the extracellular loop 2, between TMD 4 and 5, may also be involved [29-31] (Fig. 4).

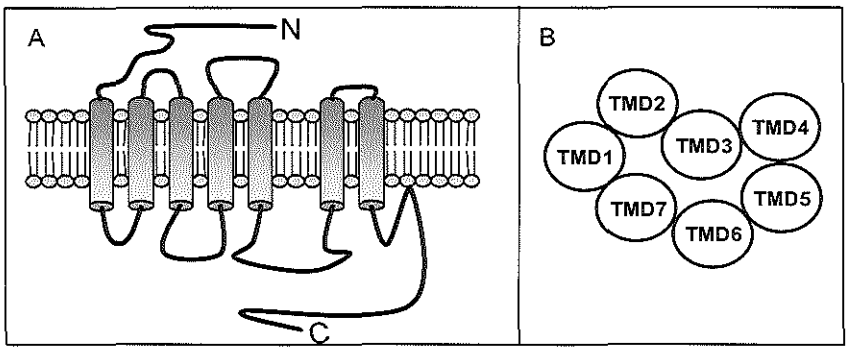

Figure 4. Putative configuration of somatostatin receptors. (A) Side view showing the seven membrane spanning α-helices. (B) Top view showing the possible configuration of the seven transmembrane (TMD1-TMD7) within the membrane.

Five distinct genes encoding SSTR have been identified and this revealed a more extensive receptor family than previously suspected from pharmacological and biochemical criteria [32-34]. Genes for SSTR1, 3, 4, 5 lack classical introns. The SSTR2 gene displays a cryptic intron at the 3' end of the coding segment, which gives rise to two splice variants, a long (SSTR2A) form and a short (SSTR2B) form [35,36]. The 2A and 2B variants differ only in the length of the cytoplasmic tail. The SSTR genes are all located on different chromosomes, but have a high degree of sequence homology, suggesting that they are derived from a common ancestor gene [37].

Expression of SSTR subtypes

SSTR are widely expressed in many tissues and frequently multiple subtypes can be present in certain cell types [38-41]. The pattern of expression of individual SSTR has been studied at the mRNA level, mainly in rodent tissues, using various techniques [33-35,42-44]. All five SSTR mRNAs are present in brain and pituitary gland. Besides the brain, the five SSTR mRNAs are variably expressed in peripheral tissues [33-35,42].

The generation of a SSTR2 knockout model showed that SSTR2 knockout mice are refractory to growth hormone-negative feedback on arcuate neurons [45]. A later study showed that SST inhibition of glucagon release in mouse islets is primarily mediated via SSTR2, whereas insulin secretion is regulated primarily via SSTR5 [46]. A clear phenotype or major defects have not been established thusfar in this model.

In addition to normal tissues, many human tumors, benign or malignant, express SSTR (reviewed in [32]). These tumors include pituitary tumors, carcinoid tumors, insulinomas, glucagonomas, pheochromocytoma, breast carcinoma, renal carcinoma, prostate carcinoma, meningioma and glioma. The presence of SSTR2 transcripts is most common in these tumors, followed by SSTR1, SSTR3 and SSTR4. SSTR5 expression appears to be tumor-specific, being positive in some tumors, e.g., breast, but absent in others, e.g., islet tumors [32]. Currently, little is known about the tissue-specific regulation of SSTR subtype expression. Studies of the promoter regions of SSTR genes should shed light on this issue in the near future [32,47].

Binding of somatostatin analogs to SSTR subtypes

On the basis of ligand binding studies using different synthetic somatostatin analogs, SSTR have been clustered into two different subgroups. SSTR2, SSTR3 and SSTR5 are able to bind octapeptide and hexapeptide analogs (SSTR3 with lower affinity than SSTR2 and SSTR5),

whereas SSTR1 and SSTR4 have negligible affinity for these compounds and only bind the natural forms SST-14 and SST-28 [48]. Recently, a series of non-peptide subtype-selective agonists were described [49]. These compounds bind with high affinity to each of the five SSTRs. *In vitro* experiments using these selective compounds demonstrated the role of SSTR2 in inhibition of glucagon release and SSTR5 as a mediator of insulin secretion. Both receptors regulate growth hormone release. The availability of these high-affinity subtype-selective agonists for several of the SSTRs represents a major step forward, because they facilitate the functional characterization of individual SSTR subtypes.

1.5 Somatostatin receptors in the hematopoietic system

Previously, the presence of SSTR has been demonstrated in human lymphoid tissues, lymphoid cell-lines and in peripheral blood cells [50-57]. Both SSTR2 and SSTR3 transcripts have been detected in freshly isolated human thymocytes (Ferone D, submitted). In contrast, human peripheral blood B- and T-lymphocytes express only SSTR3. Monocytes express SSTR2 upon activation e.g., by lipopolysaccharides (Lichtenauer-Kaligis EGR, submitted). Malignant hematopoietic cells have also been reported to express SSTR. For instance, SST receptors have been detected *in vivo* by scintigraphy in both T and B non-Hodgkin's lymphoma and Hodgkin's disease [51,57-59]. In addition, somatostatin receptor autoradiography, in which tissue sections are incubated with isotope-labeled somatostatin or somatostatin analogs, has been applied to demonstrate the presence of somatostatin binding sites in biopsies of malignant lymphomas [51]. Using similar methods, somatostatin receptors have been detected on acute lymphoblastic leukemia and acute myeloid leukemia [60]. Finally, receptors for somatostatin have also been detected on lymphoblastic leukemia by using fluorescently labeled SST [61].

Although SSTRs have been detected on certain normal and leukemic blood cell types, it has not previously been studied whether hematopoietic stem cells and progenitor cells express SSTR. Moreover, it was not known which SSTR subtypes are expressed on normal and leukemic hematopoietic cells. These issues are addressed in Chapters 2 and 3 of this thesis.

The bone marrow contains nerve terminals that produce multiple neuropeptides, including somatostatin [62-64]. It has been suggested that nerve fibers contribute to the regulation of blood cell production and the release of blood cells from the marrow into the circulation [65,66]. In addition, somatostatin-producing cells were detected at the interface between bone and bone marrow, in close contact with vessels [67]. Notably, hematopoietic stem cells have been shown to reside in this region [68,69].

In Chapter 2, studies are presented aimed at the action of SST on immature hematopoietic progenitor cells. It is shown that SST is a potent chemoattractant for $CD34^+$ cells and for committed progenitor cells of both the myeloid and erythroid lineages. These observations suggest that SST plays a role in homing and migration of hematopoietic cells.

Previous studies have shown that SST exerts inhibitory effects on the *in vitro* proliferation of AML cells [60]. Negative signals from SSTR have been attributed to binding and activation of protein tyrosine phosphatases (PTPase) [70-72]. Specifically, src homology (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1), previously referred to as hematopoietic cell phosphatase (HCP), has been postulated as the PTPase responsible for SSTR2-mediated

inhibition [70]. The antiproliferative effects of SST on AML cells are most prominent when proliferation of AML cells is stimulated with granulocyte colony-stimulating factor (G-CSF). In contrast, little or no inhibition of IL-3 or GM-CSF-induced proliferative responses is seen [60].

In Chapter 4, two studies are presented that provide an explanation for the selective inhibition of G-CSF-induced proliferation of AML cells via recruitment of SHP-1 to SSTR2.

1.6 Signal transduction pathways coupled to SSTR

The signal transduction pathways coupled to SSTR have thus far been studied mainly in transfected cell systems. Major mechanisms implicated in SSTR signaling include, G protein-linked modulation of adenylyl cyclase, Ca^{2+} and K^+ channels, Na^+/H^+ antiporter, guanylyl cyclase, phospholipase C, phospholipase A2, MAP kinase (MAPK), and serine, threonine, and phosphotyrosine phosphatases (PTP) [32-34] (Table 1). All five subtypes have been found to be linked to adenylyl cyclase via inhibitory G proteins ($G\alpha_i$), which are pertussis toxin sensitive. While the inhibitory effects on adenylyl cyclase activity and on the influx of Ca^{2+} are linked to inhibition of secretion processes, the activation of PTP or MAP kinase activity may play a role in the regulatory effects that SST may exert on cell proliferation (Fig. 5).
Using mutational analysis, transfection studies, immunoprecipitation and affinity purification techniques, a number of G proteins associated with the different SSTR have been identified [73-76] (Table 2).

SSTR signaling has thusfar been studied mainly in non-hematopoietic cell line systems. However, intracellular signaling function may depend significantly on the cellular context. Signaling properties of SSTR in hematopoietic cells have not previously been investigated. Studies dealing with the signaling compounts of SSTR2 in hematopoietic cells in relation to induction of cell migration are presented in Chapter 5.

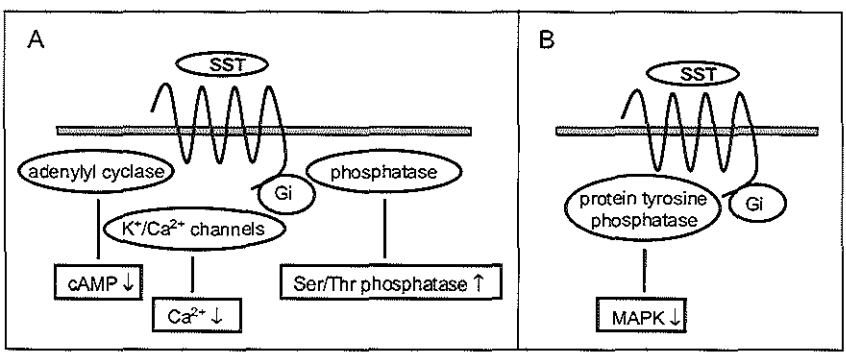

Figure 5. Schematic representation of SSTR signaling pathways leading to inhibition of secretion (A) and cell proliferation (B).

Effector coupling	SSTR1	SSTR2	SSTR3	SSTR4	SSTR5
Adenylyl cyclase activity	-	-	-	-	-
Protein tyrosine phosphatase activity	+	+	+	+	
Ca^{2+} channels	-	-			
K^+ channels		+	+	+	+
MAPK activity	+	-	+/-	+	-
Na^+/H^+ exchanger	-				
G protein coupling	$G\alpha_{i3}$	$G\alpha_{i1}$	$G\alpha_{i1}$		
		$G\alpha_{i2}$	$G\alpha_{i2}$		
		$G\alpha_{i3}$	$G\alpha_{i4}$		
		$G\alpha_{o2}$	$G\alpha_{i6}$		

Table 2. Major intracellular signaling pathways coupled to somatostatin receptors (SSTR).
Derived from reference: [25]
-: negative effect, +: positive effect.

1.7 Regulation of SSTR function

Desensitization and internalization
A common property of most G protein-coupled receptors (GPCRs) is their ability to regulate their responsiveness to continued agonist exposure [77]. Such agonist-specific regulation typically involves receptor desensitization (homologous desensitization) due to uncoupling from G proteins, as well as receptor internalization and receptor degradation [77]. Additionally, agonist-independent or heterologous desensitization may occur when hormonal activation of one receptor reduces cellular responsiveness through a different receptor system [78]. Whereas homologous desensitization may be mediated either by G protein receptor kinases (GRKs) or by second messenger-dependent kinases, heterologous desensitization is thought to involve only the latter mechanism. GRK phosphorylation promotes the binding to β-arrestin, which disrupts receptor to G protein coupling and may also act as an adapter molecule linking the receptor to clathrin-mediated endocytosis [77,79,80]. The endocytosed receptor is either sorted to the lysosomes for degradation if agonist stimulation is prolonged, or recycled back to the cell surface as a result of pH-dependent conformational change and dephosphorylation via a membrane-associated GPCR phosphatase located in the endosomes [77,79,80].

SSTR2, 3, 4 and 5 are readily internalized after ligand binding [81-84]. In contrast, SSTR1 receptors do not internalize but instead accumulate at the membrane during continued agonist treatment [83,85]. Internalization of SSTR receptors depends on critical residues in the C-tail [81,82,84,86,87]. In the C-terminus of SSTR5, both negative and positive endocytic signals have been identified [84]. Like other SSTR receptors, the C-tail of SSTR2 is rich in serine and threonine residues. Phosphorylation of cytoplasmic serine and threonine residues, especially in the carboxyl terminus of SSTR2, plays an important role in agonist-induced desensitization and internalization [82]. Recently, it was shown that C-terminally truncated rat SSTR2 (Δ349) leads to induction of agonist-independent internalization and activation [87]. Strikingly, deletion mutants of SSTR2 that are slightly larger or shorter than the Δ349 mutant did not

show constitutive internalization, indicating that the C-terminus of SSTR2 comprises multiple domains controlling receptor endocytosis.

Internalization and intracellular trafficking of GPCR may have a major influence on signaling. Studies presented in Chapter 5 have been performed to address whether internalization of SSTR2 is essential for signaling function in hematopoietic cells.

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Chapter 2

Somatostatin is a Selective Chemoattractant for Primitive ($CD34^+$) Hematopoietic Progenitor Cells

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Experimental Hematology, in press

Abstract

Objective. Somatostatin (SST) is a regulatory peptide with a wide variety of activities in different tissues. SST activates $G_{\alpha i}$-protein-coupled receptors of a family comprising five members (SSTR1-5). Despite the broad use of SST and its analogs in clinical practice, the spectrum of activities of SST is incompletely defined. Here, we examined the role of SST and its receptors in hematopoiesis.

Materials and Methods. SSTR expression on human and mouse hematopoietic cells was analyzed by flow cytometry and reverse transcriptase-polymerase chain reaction. The effects of SST on cell migration were measured in transwell assays. Using selective inhibitors, signaling mechanisms involved in SSTR2-mediated migration were studied in 32D cell transfectants expressing SSTR2.

Results. Human hematopoietic cells exclusively expressed SSTR2, whereas mouse bone marrow cells expressed SSTR2 and SSTR4. SSTR levels were high on primitive ($CD34^+$, Lin^-) but low or absent on more mature ($CD34^-$, Lin^+) cell types. Both SST and its analog octreotide acted as chemoattractants for primitive hematopoietic cells. Despite the presence of SSTR4, bone marrow cells from SSTR2 deficient mice failed to migrate towards SST gradients, suggesting that SSTR2 and SSTR4 are functionally different in this respect. SST activated phosphatidylinositol 3-kinase and the MAP kinases Erk1/2 and p38 in 32D[SSTR2] cells. While chemical inhibitors of these kinases had some effect, SST-induced migration was most strongly affected by blocking $G_{\alpha i}$ activity or by elevating intracellular cAMP levels.

Conclusion. Somatostatin acts as a selective chemoattractant for immature hematopoietic cells via activation of multiple intracellular pathways.

Introduction

The development of hematopoietic stem cells and progenitor cells towards the different types of functional blood cells is controlled by a network of regulatory growth factors and cytokines and by bone marrow stromal cells. The latter provide an appropriate microenvironment for hematopoiesis [1-4]. Increasing evidence suggests that the neuroendocrine system also influences blood cell development and function. For instance, nerve fibres present in hematopoietic tissues have been suggested to be involved in the retention of progenitor cells, the control of blood cell production, and the release of blood cells into the peripheral blood circulation [5-7]. In addition, several neuropeptides and neurotransmitters, e.g., substance P (subP), the neurokinin-1 receptor agonist NK-1, calcitonin gene-related peptide (CGRP) and noradrenalin (NE), have been shown to modulate hematopoiesis. Whereas SubP, NK-1 and CGRP significantly stimulate in vitro proliferation of hematopoietic progenitors, NE inhibits colony formation from total bone marrow cells in response to GM-CSF [8]. Finally, nerve growth factor (NGF) enhances mast cell colony formation from murine BM cells, promotes neutrophil survival and function, contributes to differentiation of human basophils and stimulates the release of inflammatory substances from these cells [9,10].

Somatostatin (SST), a regulatory peptide produced by neuroendocrine inflammatory and immune cells, was originally discovered as a peptide inhibiting the release of growth hormone from the pituitary gland [11]. SST exerts a variety of activities on multiple tissues. For instance, it modulates neurotransmission in the central nervous system and inhibits secretory and proliferative processes in many organs [12]. Two biologically active forms of SST, containing 14 and 28 amino acids, respectively, have been identified. SST-28 is a congener of SST-14, extended at the N-terminus [13]. SST inhibits the proliferation of malignant lymphoid and myeloid cells *in vitro* [14-16]. SST-producing cells are present at the interface between bone and bone marrow, a location were the most primitive hematopoietic cells reside [17]. Whether SST influences hematopoietic stem cell and progenitor cell function has not been studied.

The somatostatin receptors (SSTR) belong to a family of G-protein-coupled receptors that comprises five members. SSTR subtypes share a high degree of structural conservation. They show overlapping as well as distinct functional properties, for example with regard to SST binding and kinetics of receptor internalization [18-20]. SSTR's inhibit adenylyl cyclase and cAMP production via association with $G_{\alpha i}$-proteins [21]. Several $G_{\alpha i}$-coupled receptors have been implicated in the control of leukocyte migration [22], including the receptor for stromal cell-derived factor-1 (SDF-1), a potent inducer of migration of hematopoietic progenitor cells, lymphocytes and monocytes [23,24]. Here, we show that SSTR are specifically expressed on immature ($CD34^+$) human and murine hematopoietic progenitors and that SST acts a chemoattractant for these cells. By using a 32D cell model expressing SSTR, we demonstrate that SST-induced migration of hematopoietic cells involves multiple cooperating intracellular signaling pathways.

Materials and Methods

Isolation of human hematopoietic cells
Human bone marrow (BM) and mobilized peripheral blood (PB) progenitor cells were obtained from healthy donors following informed consent. BM cells were collected in heparinized Hank's Balanced Salt Solution (HBSS; GIBCO, Breda, The Netherlands). Mononuclear cells were collected after Ficoll-Isopaque density centrifugation [25]. $CD34^+$ selections were performed on a CEPRATE SC (CellPro Inc, Bothell, WA) as previously described [26].

Mouse bone marrow
SSTR2 deficient mice [27] and control littermates were used at the age of 12 to 16 weeks. BM cells were obtained from femurs and tibias and suspended in HBSS with 5% fetal calf serum (HBSS/FCS). After passing through a 100 μm sieve, cells were spun down and resuspended, resulting in monocellular suspensions containing 98-99% viable cells, as determined by trypan blue exclusion. To obtain enriched precursor cell fractions, BM cells of 4-6 mice were pooled and suspended in HBSS/FCS and subsequently incubated at 37°C for 1 hr in a 162 cm^2 tissue culture flask (Costar, Cambridge, MA) to remove monocytes. Nonadherent cells were resuspended in 45% (ρ = 1.058) Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) in Ca^{2+}/Mg^{2+}-free HBSS and loaded onto a Percoll density gradient as described [28]. Precursor cells were collected from the interface between the 1.058 and 1.064 density layers and washed twice in Ca^{2+}/Mg^{2+}-free HBSS supplemented with 0.2% BSA (HBSS/BSA).
Detection of SSTR by RT-PCR
Reverse transcriptase-polymerase chain reactions (RT-PCR) were performed as described [29]. Oligonucleotide primers specific for human and mouse SSTR1-5 receptor subtypes are listed in Table 1.

Flow cytometric analysis of SSTR expression in BM subsets
Cells were incubated with 50 nM of Fluo-somatostatin™ (Fluo-SST; Advanced Bioconcept, Montreal, Quebec, Canada) in phosphate buffered saline (PBS) and 1% FCS for 45 min at room temperature (RT) in the dark. Fluo-SST binds to SSTR1-3 but not to SSTR4 and SSTR5. Non-specific binding was assessed in control incubations containing a 100-fold excess of (D-Trp^8)-somatostatin-14 (BACHEM AG, Bubendorf, Switzerland). Human BM cells were co-stained with R-phycoerythrin (PE)-conjugated antibodies (Beckman Dickinson, San Jose, CA). This panel included CD34-PE, CD117-PE, CD14-PE, CD4-PE, CD8-PE, CD19-PE, CD33-PE and IgG_1-PE (negative control). For three-color analysis, cells were stained with Fluo-SST, CD117-PE and a Cy5-conjugated anti-CD34 (CD34-Cy5). Mouse BM cells were co-stained with rat-anti-mouse MoAbs anti-CD34 (MEC14.7, Sanbio BV, Uden, The Netherlands), Sca-1 (E13161-7; [30]), ER-MP58 [31], Gr-1 (RB68C5; [32]), or a mixture of anti-CD3 (KT3; [30]), Mac-1 (M1/70; [33]) and B220 (Ra3-6B2; [34]). Goat anti-rat-Ig coupled to PE (Caltag Laboratories, Burlingame, CA) was used as the second antibody. To detect murine CD117, cells were incubated with biotin-kit ligand (KL; 1:20 vol/vol; Immunex, Seattle, WA) for 1 hr at 4°C and streptavidin-phycoerythrin (SA-PE, 1:200 vol/vol, Caltag Laboraties, Burlingame, CA) for 30 min at 4°C. Specificity of biotin-KL binding was determined in control incubations with MoAb ack2, a reagent that interferes with KL binding to CD117 [35]. For three-color detection of SSTR, CD117 and CD34 or Sca-1, cells were first incubated with Fluo-SST and biotin-KL and then with streptavidin-allophycocyanin (SA-APC, Becton-Dickinson, San Jose, CA) to detect bound biotin-KL. Subsequently, cells were incubated with either rat MoAb against mouse CD34 or anti-Sca-1, followed by goat anti-rat-

phycoerythrin (PE) conjugate. Flow cytometric analysis was performed using FACScan and FACS Calibur equipment (Becton-Dickinson, Sunnyvale, CA).

human	Sequence (5'- 3') *	Position †	PCR product
hSSTR1 -FW ‡	ATGGTGGCCCTCAAGGCCGG	754	318 bp
hSSTR1 -RV ‖	CGCGGTGGCGTAATAGTCAA	1071	
hSSTR2 –FW ¶	GCCAAGATGAAGACCATCAC	214	414 bp
hSSTR2 -RV	GATGAACCCTGTGTACCAAGC	627	
hSSTR3 -FW	CCAACGTCTACATCCTCAACC	236	314 bp
hSSTR3 -RV	TCCCGAGAAGACCACCAC	549	
hSSTR4 -FW	ATCTTCGCAGACACCAGACC	547	321 bp
hSSTR4 -RV	ATCAAGGCTGGTCACGACGA	867	
hSSTR5 -FW	CGTCTTCATCATCTACACGG	596	226 bp
hSSTR5 -RV	CCGTCTTCATCATCTACACGG	819	
mouse	Sequence (5'- 3') *	Position †	PCR product
mSSTR1-FW	GTGATGATGGTGGTGATGGT	817	259 bp
mSSTR1-RV	TCAGGGCAGTGGCATAGTAG	1075	
mSSTR2-FW ¶	ATCATCAAGGTGAAGTCCTCTG	694	376 bp
mSSTR2-RV	GGGTCTCCGTGGTCTCATT	1069	
mSSTR3-FW	CTCGTGTCAGTGGGTACAGG	732	297 bp
mSSTR3-RV	CGACGTGATGGTCTTAGCAG	1028	
mSSTR4-FW	CTAGGACTGGCTCCAAGGAC	1317	299 bp
mSSTR4-RV	AGACCGACACAGAGGGAAAC	1615	
mSSTR5-FW	GCTCAGAACGCAAGGTGACT	722	268 bp
mSSTR5-RV	GCATCCTCCACACCGTATC	989	

Table 1. Primers used in RT-PCR analysis of SSTR subtype expression.
* The sequences of the primers for hSSTR1 and hSSTR4 are derived and adapted from Wulfsen et al. [63], for hSSTR5 from Kubota et al. [64]. All other primers were designed by use of the Primer3! software (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) and the appropriate GenBank entries. † The position is given of the 5' nucleotide of the primer relative to the first nucleotide of the coding region in the cDNA sequence. ‡ FW: forward primer. ‖ RV: reverse primer. ¶ The SSTR2 primers are specific for SSTR2A.

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). In migration experiments with primary cells, the pore filters were pre-coated overnight at 4°C with 20 μg/ml fibronectin (Bovine plasma: 0.1% in 0.5M NaCl/ 0.05 M Tris pH 7.5, Sigma F 1141). Cells (1-2 x 10^5) 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 (Oct, Novartis Pharma, Basel, Switzerland), (D-Trp^8)-somatostatin-14 (Trp-SST) or SDF-1 (Sanvertech, Heerhugowaard, the Netherlands), was placed in the lower chamber of the transwell system. In some experiments, cells were pretreated for 30 min with oct (10^{-6} M) or SDF-1 (100 ng/ml). Chambers were maintained at 37°C, 5% CO_2 for 4 hours. Cells that had migrated into the lower chamber were counted using a cell counter (CASY®1/TTC, Schärfe Systems GmbH, Reutlingen, Germany) and in some experiments plated in *in vitro* colony assays.

In vitro colony assays

To assay for granulocyte macrophage precursors (CFU-GM), human $CD34^+$ cells were plated in triplicate in 35 mm Petri dishes (Falcon, Becton Dickinson, Oxford, UK) containing 1 ml methylcellulose medium (Methocult M3230; Stem cell Technologies, Incorporated, Vancouver, BC), 25 ng/ml human interleukin-3 (hIL-3; Genetics Institute, Cambridge, MA), 100 ng/ml human granulocyte colony stimulating factor (G-CSF; Amgen Incorporated,

Thousand Oaks, CA), 100 U/ml human granulocyte-macrophage-CSF (hGM-CSF; Immunex, Seattle, WA). Erythroid burst-forming units (BFU-E) were cultured in quadruple in 24 wells plates (Costar Corning Incorporated, Corning, NY) in 250 µl Methocult M3230 (Stem cell Technologies Incorporated) supplemented with 2.5 ng/ml hIL-3 and 1 U/ml erythropoietin (EPO; Janssen Cilag). CFU-GM and BFU-E were counted on day 14 of culture. Mouse colony-forming units-culture (CFU-C) were cultured in triplicate in 35 mm dishes containing 1 ml Methocult M3230, 30% fetal bovine serum (FBS), 1% BSA, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine with 100 ng/ml G-CSF (Amgen), 20 U/ml murine GM-CSF (Dr Steven Neben, Genetics Institute, Cambridge, MA) plus 10 µg/ml murine stem cell factor (SCF). Colonies (50 cells or more) were counted on day 7 of culture.

32D cells and transfections

The IL-3-dependent murine myeloid cell line 32Dcl10 [36] was maintained in RPMI 1640 medium supplemented with 10% FCS and 10 ng of murine IL-3 per ml at 37°C and 5% CO_2. Human SSTR2 cDNA (a kind gift from Dr G.I. Bell, Howard Hughes Medical Institute, Chicago IL) was cloned into the *Bam*HI/*Sal*I site of the eukaryotic expression vector pBABE [37]. 32D cells were electroporated with 10 µg *Pvu*I-digested pBABE[SSTR2] or empty plasmid, using a Progenitor apparatus set at 230 V, 100 µF, and 1 sec. After 48 hrs, transfected cells were selected on culture medium containing puromycin (1 µg/ml). Multiple clones were expanded for further analysis. To interfere with specific signaling pathways the following chemicals were used: MEK inhibitor U0126 (Alexis Biochemicals, Kordia Laboratory Supplies, Leiden, The Netherlands); PI3-K inhibitors LY294002 (Biomol) and Wortmannin (Sigma), p38 MAPK inhibitor SB203580 (Alexis Biochemicals), dibutyryl cAMP (dbcAMP, Biomol), Src kinase inhibitor pp2 (Sigma), and the $G_{\alpha i}$ inhibitor pertussis toxin (Sigma).

Cell lysates and Western blotting

Western blotting and preparation of cell lysates were performed as described [38]. Antibodies used for Western blotting were α-Erk1 and α-phospho-Erk (Tyr204) (Santa Cruz Biotechnology Inc, Santa Cruz, CA), α-p38 and α-phospho-p38 (New England Biolabs, Inc. Beverly, MA), α-protein kinase B (PKB/Akt) (New England Biolabs) and α-phospho-PKB/Akt (Ser473) (New England Biolabs).

Results

Expression of SSTR on human and mouse hematopoietic cells

In RT-PCR analysis with specific primer sets for the five human SSTR subtypes, only SSTR2 could be detected (Fig. 1A). Flow cytometric analysis with Fluo-SST showed that SSTR expression is restricted to $CD34^+$ BM cells. The $CD34^+/CD117^+$ subset, comprising the most immature population of $CD34^+$ cells, showed the highest SSTR expression (Fig. 1B). Fluo-SST also bound to purified $CD34^+$ peripheral blood (PB) and BM cells (Fig. 1C). BM subsets positive for lineage markers CD14 (monocytic), CD4/CD8 (T-cell), CD19 (B-cell) and CD33 (myelocytic) did not stain with Fluo-SST (Table 2), indicating that SSTR levels are high on primitive hematopoietic progenitors and decline with progressive differentiation to myeloid and lymphoid lineages. RT-PCR analysis showed that mouse BM progenitor cells express both SSTR2 and SSTR4 (Fig. 2A). As expected, only SSTR4 transcripts were detected in BM cells of SSTR2-deficient mice (Fig. 2A). Similar to human BM, Fluo-SST bound to immature ($CD34^+$, $CD117^+$, $Sca\text{-}1^+$) cells (Fig. 2B and 2C) but not to B-cells, T-cells and differentiated

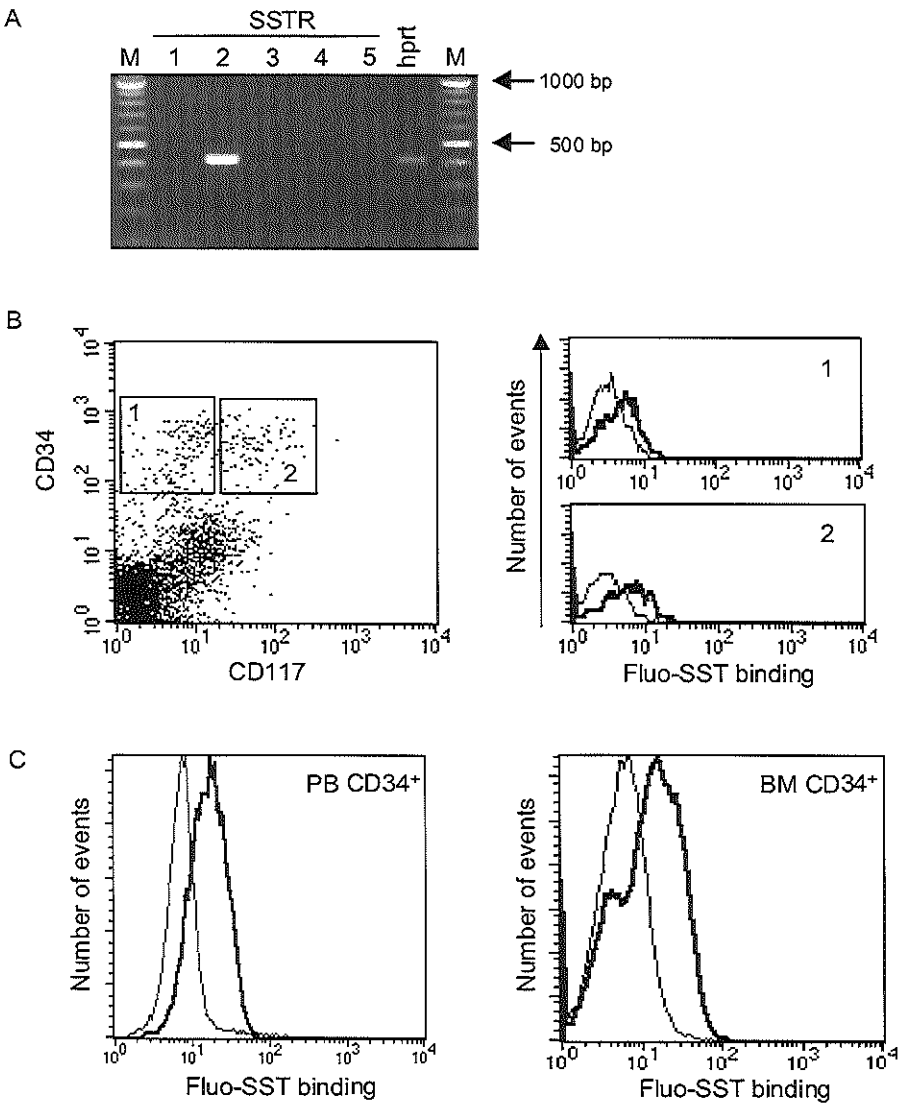

Figure 1. SSTR expression on human hematopoietic cells. *(A)* RT-PCR analysis of SSTR subtypes. PCR with hprt specific primers served as a positive control. Mock-reverse-transcribed samples and controls without template did not yield PCR products (not shown). Marker lanes (M) contain a 100 bp DNA ladder. *(B)* Fluo-SST binding to human $CD34^+$ cells. Window 1 was set to select $CD34^+/CD117^-$ cells, window 2 to select $CD34^+/CD117^+$ cells. Cells were incubated in the absence (solid line) or presence (thin line) of a 100-fold excess of $(D\text{-}Trp^8)$-somatostatin-14 (right panel). No specific binding of Fluo-SST to $CD34^-$ cells was seen *(C)* FACS analysis of Fluo-SST binding to purified PB $CD34^+$ and BM $CD34^+$ cells.

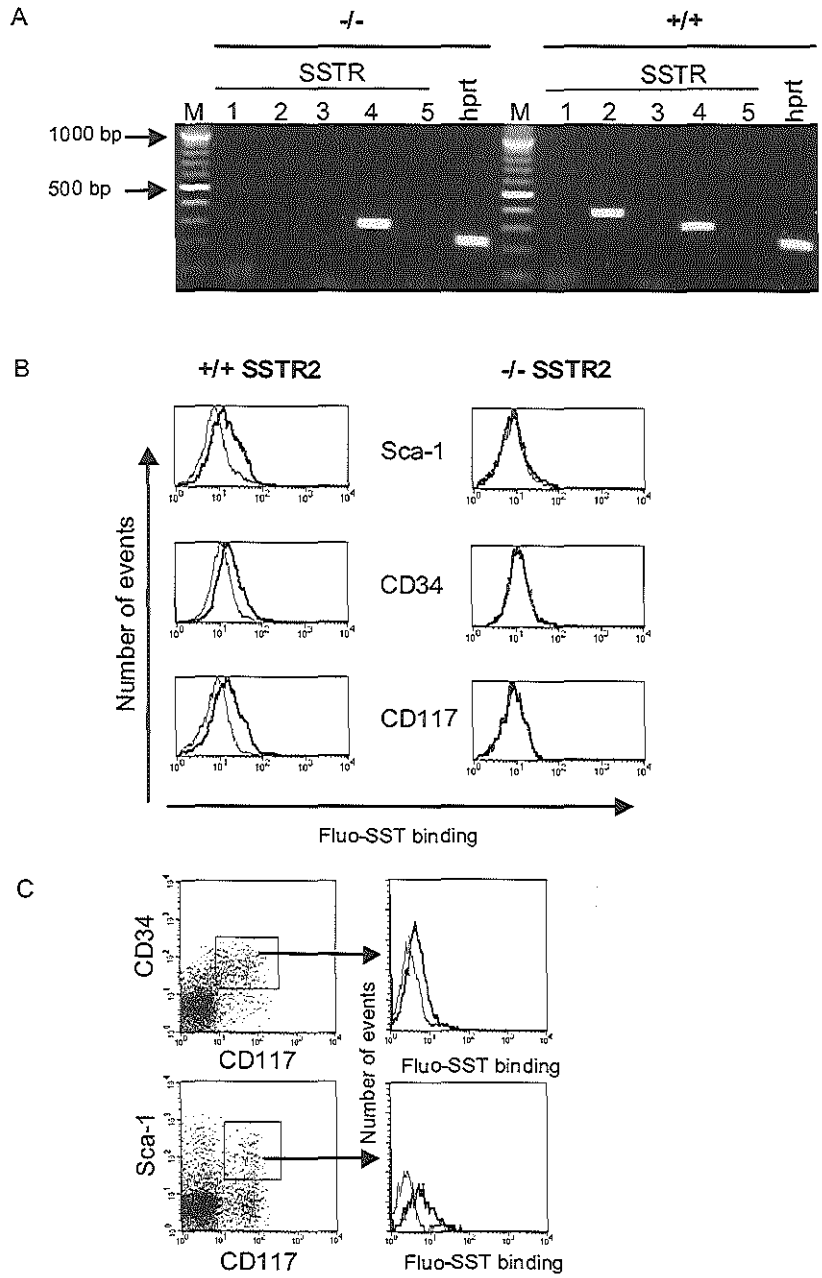

Figure 2. SSTR expression on mouse BM cells. *(A)* RT-PCR analysis in bone marrow from wild-type (+/+) and SSTR2 knockout (-/-) mice. For further details see Fig. 1A. *(B)* Fluo-SST binding to +/+ and -/- BM cells. Histograms represent Fluo-SST binding to cells gated for Sca-1, CD34 or CD117 expression. Cells were analyzed as outlined under Fig. 1B. *(C)* Fluo-SST binding to $CD34^{+}/CD117^{+}$ and $Sca1^{+}/CD117^{+}$ cells. Histograms show SST binding as outlined in Fig. 1B for indicated windows.

myelomonocytic cells (Table 2). Despite the fact that BM cells from SSTR2 (-/-) mice express SSTR4 transcripts, the cells did not stain with Fluo-SST (Fig. 2B). It must be noted, however, that this does not imply that SSTR4 protein is not expressed on mouse BM cells, because Fluo-SST does not bind to SSTR4.

cell type	marker **	Fluo-SST binding ††
Stem cells	CD34, *CD34*	+
and primitive	CD117, *CD117*	+
progenitor cells	*Sca-1*	+
	Thy-1 low	+
	CD34+CD117,	++
	CD34+CD117	
	Sca-1+CD117	++
	ER-MP58	+
B- and T-cells	CD19, *B220*	-
	CD4+CD8, *CD3*	-
Monocytes and granulocytes	CD33, CD14, *Gr-1, Mac-1*	-

Table 2. Fluo-SST binding to human and mouse BM subsets.
** Human markers are indicated in normal and mouse markers in italic lettertypes.
†† Determined by FACS analysis as outlined in Material and Methods. (-) no binding, (+) intermediate binding and (++) high binding.

SST and oct induce migration of immature progenitor cells

SST inhibits proliferation of various malignant hematopoietic cell types [15,16,39]. In contrast, SST exerted no significant anti-proliferative effects on normal hematopoietic progenitors in hematopoietic growth factor (G-CSF, GM-CSF or IL-3)-containing colony cultures (data not shown). Next, we investigated the effects of SST and oct on migration of total human or mouse BM cells in transwell migration assays. No significant migration was observed (data not shown), but this was not unexpected in view of the low percentage of cells expressing SSTR2. In contrast, bone marrow and peripheral blood-derived $CD34^+$ cells showed significant migration in response to oct, at optimal concentrations between 10^{-11} and 10^{-9} M (Fig. 3A and 3B). Migration was reduced to basal levels when the gradient was reverted by placing oct (10^{-9} M) with the cells in the upper chamber (Fig. 3B), indicating that oct predominantly induces chemotaxis instead of chemokinesis. Clonogenic myeloid progenitors (CFU-GM) and erythroid progenitors (BFU-E) also showed a chemotactic response towards oct (Fig. 3C and 3D). Similar results were obtained with low density mouse BM cells fractionated by Percoll gradient (Fig. 4). Significantly, SST and oct did not induce migration of BM cells and CFU-Cs from SSTR2 (-/-) mice, despite the presence of SSTR4 in mouse BM (Fig. 4A-C). These findings underscore the specific involvement of SSTR2 in migration of both human and murine hematopoietic progenitor cells. We have no data to explain the lower sensitivity of murine progenitors to octreotide compared to human progenitors in the migration assay. Since the FACS analysis showed approximately the same level of SSTR expression on human and mouse BM cells, we consider differences in affinity for the synthetic ligand octreotide as the most likely explanation for this discrepancy.

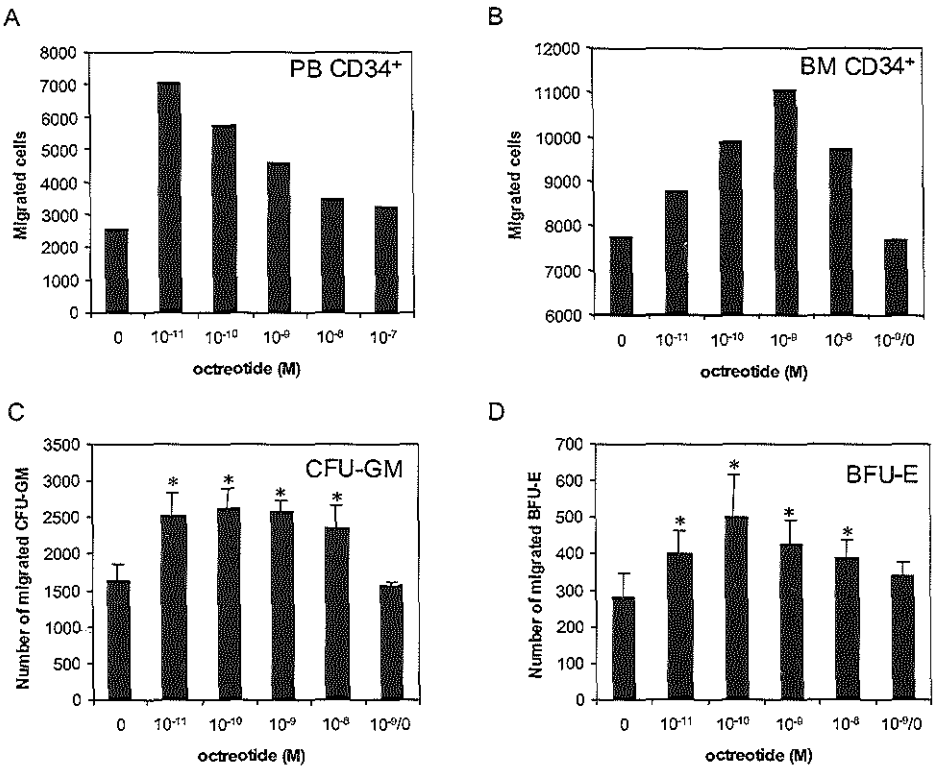

Figure 3. Migration of human progenitor cells in response to oct. *(A)* PB $CD34^+$ and *(B)* BM $CD34^+$ cells. *(C)* CFU-GM and *(D)* BFU-E. To introduce a negative gradient oct (10^{-9} M) was added to the upper chamber (10^{-9}/0, panels *B, C* and *D*). Representative graphs from three independent experiments are shown. The asterisk (*) indicates statistical significant migration compared with control (without octreotide) at P values <0.05.

Comparison of SST/oct-induced and SDF-1-induced responses in a 32D cell model expressing SSTR2

To study the molecular mechanism of SSTR2-mediated migration, we generated 32D cell transfectants expressing SSTR2 (32D[SSTR2]). 32D[SSTR2] bind Fluo-SST (Fig. 5A) and migrate in response to oct (Fig. 5B) and Trp-SST (Fig. 5C). Maximal responses occurred at somewhat lower concentrations (10^{-12} to 10^{-13} M) than seen with primary $CD34^+$ cells, which might be due to the higher levels of SSTR2 expression on the 32D[SSTR2] cells (compare Figs 1C, 2C and 5A). Similar to $CD34^+$ BM cells, 32D[SSTR2] cells showed a strong migratory response towards positive gradients, indicative of chemoattraction, and no or little migration in, respectively, a negative or an absent gradient (Fig. 5D). No migration was observed with SSTR negative parental 32D cells (Fig. 5A and 5D).

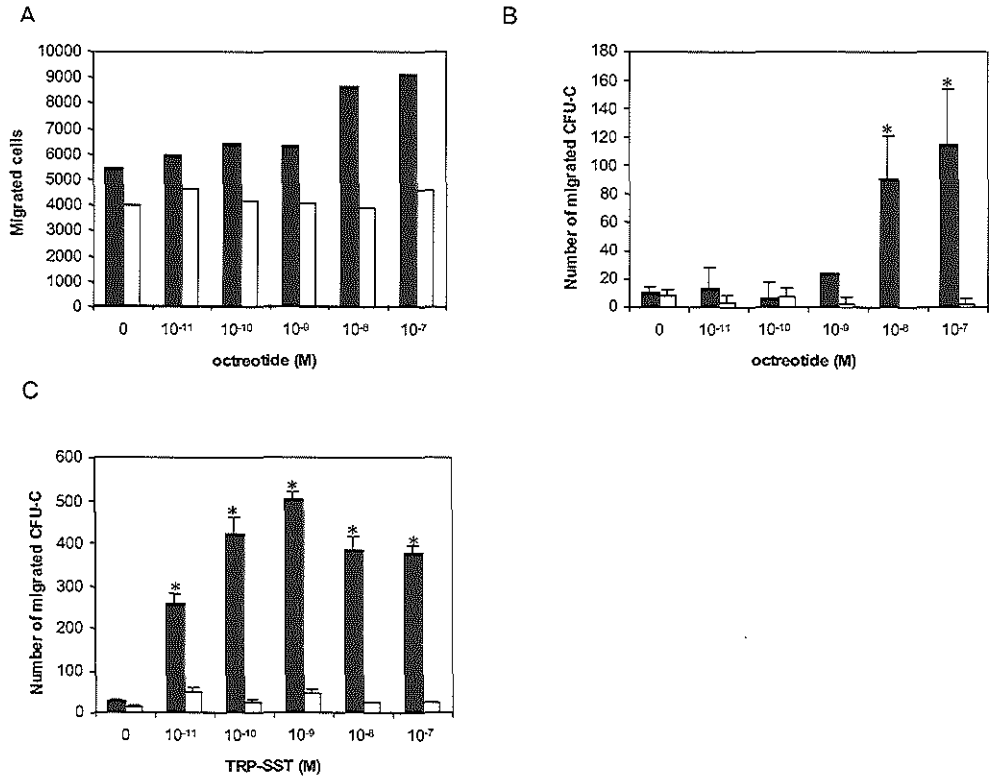

Figure 4. Migration of murine BM cells in response to oct (*A* and *B*) or Trp-SST (*C*). Black bars: SSTR2 +/+ cells. Open bars: SSTR2 -/- cells. *(A)* Percoll fractionated low density BM cells; *(B)* and *(C)*: CFU-C. Representative graphs from three independent experiments are shown. The asterisk (*) indicates statistical significant migration compared with control (without octreotide or TRP-SST) at P values <0.05.

Oct induced migration of 32D[SSTR2] cells with a similar efficiency (approximately 30%) as SDF-1 (Fig. 6). As expected, pretreatment of cells with the respective ligands prevented the cells to migrate towards a positive gradient. Strikingly, pretreatment of 32D[SSTR2] cells with oct also almost completely antagonized SDF-1-induced migration and *vice versa* (Fig. 6). The magnitude of SST-induced migration of CD34 cells is approximately 27-44% of SDF-1-induced responses as shown in two independent peripheral blood CD34 samples (Table 3). Similar to the 32D model, SDF-1 in the upper chamber inhibits SST-induced migration. Conversely however, no competitive effects of SST on SDF-1-induced migration of primary $CD34^+$ cells were detectable. Clearly at equimolar concentrations, SDF-1 acts dominantly over SST, which might be due to differences in expression levels of CXCR4 and SSTR2 on $CD34^+$ cells.

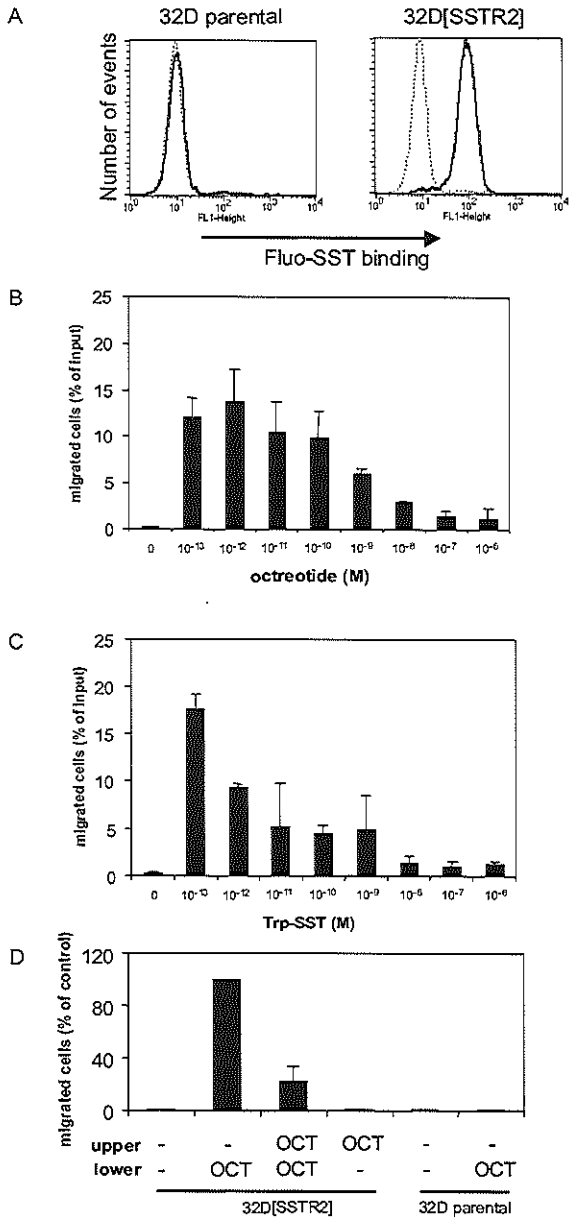

Figure 5. *(A)* Fluo-SST binding to 32D parental and 32D[SSTR2] cells. For details see Fig. 1. *(B)* Migration of 32D[SSTR2] cells in response to increasing concentrations of oct or *(C)* Trp-SST. *(D)* Chemotactic versus chemokinetic activity of oct. Oct (10^{-9} M) was added to upper and/or lower chambers as indicated. Parental 32D cells were taken along as a control. Data are expressed as the percent of migration to a positive gradient (100%) and represent means ± SE.

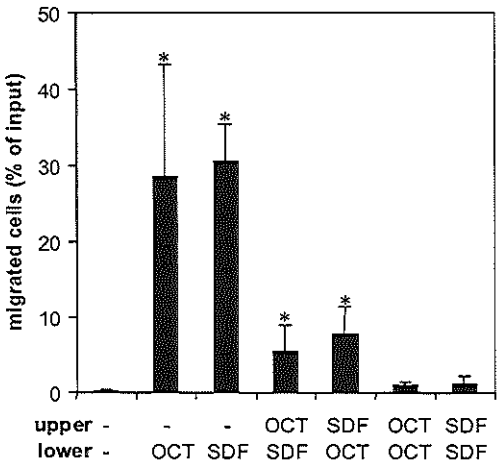

Figure 6. Cross-inhibitory effects of SDF-1 or oct on 32D[SSTR2] cell migration. SDF-1 (100 ng/ml), oct (10^{-9} M), or control medium (-) were added to the upper or lower chamber as indicated. The asterisk (*) indicates statistical significant migration compared with control (without octreotide or SDF-1) at P values <0.05.

lower	upper	PB 1 number	PB 1 %‡‡	PB 2 number	PB 2 %‡‡
-	-	4740	15.9	2835	26.0
oct	-	8040	27.0	4830	44.2
SDF	-	29800	100	10920	100
SDF	oct	35000	117	11500	105
oct	SDF	2505	8.4	1635	15.0
oct	oct	4005	13.4	3285	30.1
SDF	SDF	9525	32.0	2730	25.0

Table 3. Cross-inhibitory effects of SDF-1 or oct on PB $CD34^+$ cell migration.
SDF-1 (100 ng/ml), oct (10^{-9} M), or control medium (-) were added to the upper or lower chamber as indicated.
‡‡: Migration induced by SDF-1 is set at 100%.

SST activates multiple signaling pathways in 32D[SSTR2] cells

$G_{\alpha i}$-coupled receptors not only modulate cAMP levels but, depending on the type of receptor and the cellular context, also activate other signaling routes, such as the Raf/ERK, PI3-K/PKB and p38 MAPK pathways. Because these mechanisms have been implicated in the control of cell migration [40-47], we first investigated whether they are activated by SST or oct in 32D[SSTR2] cells. As shown in Fig. 7, incubation of 32D[SSTR2] cells with oct, following 4 hrs of serum and cytokine deprivation of the cells, resulted in a significant induction of Erk1/2, p38 MAPK and PKB phosphorylation (Fig. 7).

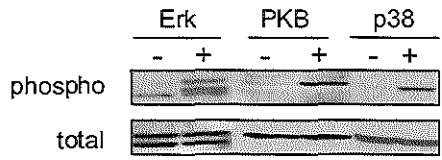

Figure 7. Oct-induced phosphorylation of Erk, p38 MAPK and PKB. After growth factor and serum deprivation (4h), cells were stimulated for 5 min with either medium alone (-) or with medium + 10^{-6} M oct (+). Western blot analysis was performed with antibodies recognizing phosphorylated and total protein as indicated.

We subsequently studied the effects of selective inhibitors of the SSTR-coupled signaling pathways on migration of 32D[SSTR2] cells. Inhibition of $G_{\alpha i}$-protein activity with pertussis toxin (PTX) [48] completely abrogated migration. A similar effect was seen of dibutyryl (db)cAMP (Fig. 8A), indicating that SSTR2-coupled $G_{\alpha i}$ activity, leading to reduced cAMP levels in the cells, is essential for migration induction. The PI3-K inhibitors LY294002 and Wortmannin reduced oct-induced migration by approximately 50% (Fig. 8B and C). In contrast, the MEK inhibitor U0126 and the p38 MAPK inhibitor SB203580 did not affect oct-induced migration (Fig. 8D and E). However, SB203580, U0126 and LY294002 did significantly inhibit migration when added in combination with dbcAMP (Table 4). These observations suggest that Erk1/2, p38 MAPK, and PI3-K-controlled mechanisms can contribute to SSTR2-mediated chemoattraction, depending on intracellular cAMP levels. Because Src-like protein tyrosine kinases have been shown to link G-protein-coupled receptors to PI3-K and MAPK routes [49], we also tested the effects of the selective Src tyrosine kinase inhibitor pp2 on SST-induced cell migration. However, pp2 did not affect SST responses (Fig 8F).

	DbcAMP (μM)			
	0	100	250	500
no inhibitor	100‖‖	60.6	33.6	23.8
U0126 (50 μM)	91.2	19	7.5	3.8
SB (25 μM)	88	4	1.8	1.8
LY (10 μM)	54.8	3.3	2.6	2.2

Table 4. Effect of MAPK and PI3-K inhibitors on SST-induced migration
‖ ‖: Data are expressed as the percentages of migration induced by octreotide (10^{-9} M).

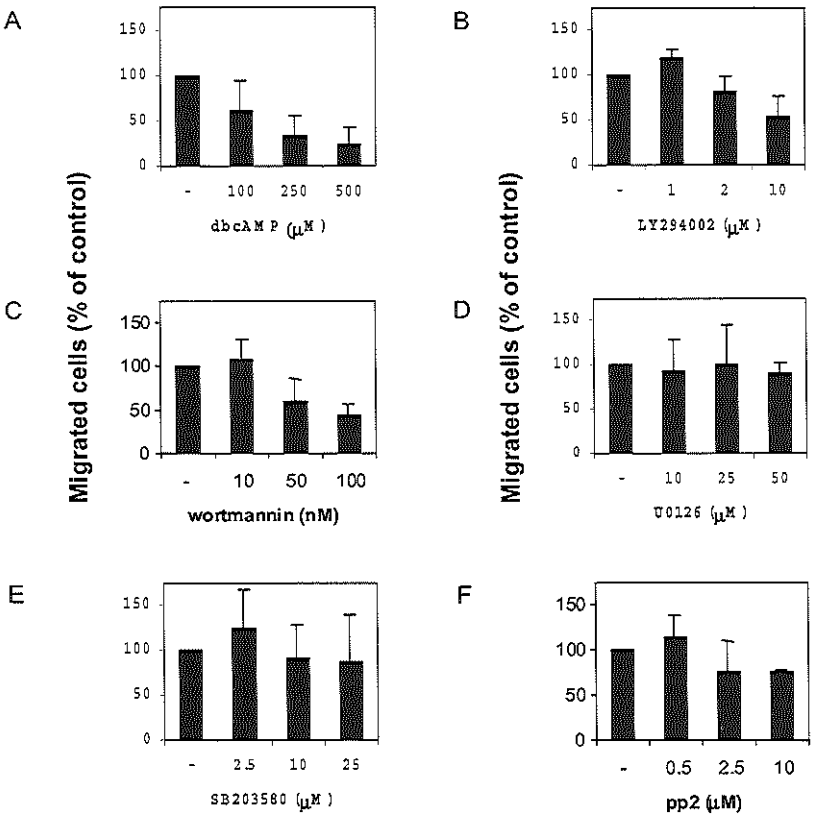

Figure 8. Effects of dbcAMP and inhibitors of p38 (SB203580) Erk (U0126) PI-3K (LY294002 and Wortmannin) and Src (PP2) activity on oct-induced migration. 32D[SSTR2] cells were pretreated for 30 min with different inhibitors at concentrations indicated. Data are expressed as the percent migration of untreated (solvent control) cells.

Discussion

SST is a regulatory peptide with a wide variety of functions, mainly linked to the neuroendocrine and immune systems [12,21]. Thus far, SST has been found to act predominantly as an inhibitor of secretory and proliferative responses. In this study, we have established that SST is a chemoattractant for primitive hematopoietic progenitor cells and thus identified a novel function for this peptide.

We found that SST-induced migration of hematopoietic progenitors is mediated exclusively via SSTR2. While SSTR2 is the only receptor subtype found in human hematopoietic cells, mouse bone marrow cells also express SSTR4, at least at the transcriptional level. Nevertheless, SST failed to attract hematopoietic cells from SSTR2-deficient mice, indicating that the role of SSTR4 in migration is negligible. This may relate to differences in the

signaling properties between SSTR2 and SSTR4 subtypes [21]. Alternatively, SSTR4 protein levels on the cell membrane might be too low for inducing migratory responses.

The process of cell migration is highly complex and involves cell polarization, formation of lamellipodia and filipodia and attachment of the cell to the surface on which it is migrating [50,51]. How the several cytokines and chemokines that induce migration of hematopoietic cells control these cellular responses is still unclear. The small molecular weight GTP binding proteins play a major role in cell adhesion and migration. For instance, the formation of filopodia is regulated by cdc24 [52,53], of lamellipodia by Rac [54], while focal adhesions are controlled by Rho [55]. In addition, focal adhesion kinase, PI3-K and phospholipase C have been implicated in the activation of cell migration [50]. We delineated some of the signaling mechanisms involved in SSTR2-mediated migratory responses. SSTR2 couples to $G_{\alpha i}$, the class of G-protein that antagonizes adenylate cyclase-mediated increase of cAMP and activation of protein kinase A (PKA) [56]. The observation that dbcAMP and PTX, which uncouples $G_{\alpha i}$-proteins from receptors, both inhibit octreotide-induced migration confirms that this pathway is crucial for this response. PKA phosphorylates RhoA, leading to inactivation of this GTPase. Because Rho is implicated in the formation of stress fibers and focal adhesions, this might be one of the mechanisms controlled by intracellular cAMP [57]. Inhibition of PI3-K activity by Wortmannin or LY294002 also significantly reduced octreotide-induced cell migration. PI3-K is implicated in the regulation of cell polarization at multiple levels, one of the critical targets being Rho [57]. In addition, PI3-K is involved in the recruitment of multiple pleckstrin homology domain-containing signaling proteins to the membrane, including nucleotide exchange factors for small GTPases, PKB and other proteins implicated in cell migration [58]. We have also shown that oct induces phosphorylation of the MAP kinases Erk1/2 and p38 (Fig 7). Although inhibitors of these kinases by itself had little effect, they significantly inhibited oct-induced migration when combined with dbcAMP. We conclude from these results that, depending on conditions determining intracellular cAMP levels, both Erk1/2 and p38 MAPK can contribute to SSTR2-mediated migration of hematopoietic cells.

The migration-inducing effects of SST/oct showed a typical "bell-shape" dose response relationship in which the induced effects are lost at higher ligand concentrations. This phenomenon is often seen in G-coupled receptor mediated responses and reflects a rapid ligand-induced uncoupling of G-proteins from the receptor, leading to prolonged receptor desensitization. Strikingly, we observed that pretreatment of cells with oct also antagonized SDF-1-induced migration and *vice versa*. There are several potential explanations for this finding, which are not mutually exclusive. First, it is possible that receptor cross-desensitization is involved, as has recently been demonstrated for a variety of chemoattractant receptors [59]. Alternatively, preincubation with heterologous ligand may have profound effects on cell polarization, and thereby affect chemotaxis.

A major question that remains is how SST affects hematopoietic stem cell development and homing in vivo. Unlike mice deficient for SDF-1 or its receptor CXCR4, which display a lethal bone marrow failure [60,61], we did not observe gross hematopoietic defects in SSTR2 knockout mice. Immunophenotypic analysis of bone marrow cells from these animals also did not reveal abnormalities in the lymphoid, myeloid and erythroid compartment and CFU-C and BFU-E contents of bone marrow of SSTR2 (+/+) and (-/-) mice were similar (data not shown). These data suggest that SST does not play a major role in the control of adult hematopoiesis under basal conditions. We studied migration of more early progenitors using the CAFC assay [62]. However, only CAFC week 1 from SSTR2 (+/+) but not SSTR2 (-/-)

mice are strongly attracted by SST. CAFC week 1 reflects a relatively mature subset of progenitors, close to CFU-C, supporting the result of Figure 4. More primitive progenitor and stem cell subsets showed no specific promigratory effects of SST (data not shown). While these results suggest that the effects of SST are restricted to relatively mature progenitors, a complication is that the frequencies of CAFC week 3, 4 and 5 are too low for adequate measurements in the migration assay. Whether SST influences migration of fetal hematopoietic stem cells or affects hematopoiesis under pathological conditions with increased levels of SST (including patients receiving long term treatment with SST analogs) requires further investigation. It is conceivable that high concentrations of SST in the bone marrow would interfere with SDF-1-controlled lodging of hematopoietic stem cells, thereby reducing stem cell activity.

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Chapter 3

Somatostatin induces migration of acute myeloid leukemia cells via activation of somatostatin receptor subtype 2

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Leukemia (2001) **15** 621-627

Abstract
Somatostatin, a neuropeptide with multiple activities, exerts its function via G-coupled membrane receptors. Five somatostatin receptor subtypes, SSTR1-5, have been identified. We have recently established that somatostatin acts as a chemoattractant on normal hematopoietic progenitor cells. Here, we studied the expression of somatostatin receptors (SSTR) on leukemic cells from 16 AML patients. Using fluorescent somatostatin (Fluo-SST) in flow cytometry, we found that SSTR are expressed in variable amounts on primary AML cells. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis and immunochemistry revealed that only SSTR subtype 2 is expressed by AML cells. Using a two-chamber in vitro migration assay, we show that AML cells migrated towards a gradient of octreotide, a stable synthetic analogue of somatostatin. The degree of migration correlated with the cell surface density of SSTR2 as measured by Fluo-SST binding. These findings indicate that somatostatin influences trafficking of AML cells, which may have implications for the distribution of AML cells in the body and for clinical applications of somatostatin and analogues thereof in the context of AML.

Introduction

Two biologically active forms of somatostatin have been identified, SST-14 and SST-28. These peptides exert a wide variety of actions, which include inhibition of secretory and proliferative processes in many target organs [1]. The effects of somatostatin are mediated via high affinity, seven-transmembrane G-protein-coupled receptors (SSTRs) [2-3]. Human SSTRs are encoded by a family of 5 genes, localized on different chromosomes [3]. Each SSTR subtype displays a marked degree of structural conservation across species. SSTRs are widely distributed among many tissues and some cell types express multiple SSTR subtypes [3-5]. The five receptors share common signaling pathways, involving the inhibition of adenylyl cyclase, activation of phosphotyrosine phosphatase (PTP), and modulation of mitogen-activated protein kinase (MAPK) [3-5]. Several biological responses to somatostatin have been identified that display absolute or relative receptor subtype selectivity. These include growth hormone secretion (induced via SSTR2 and SSTR5) , insulin secretion (SSTR5), glucagon secretion (SSTR2), and immune responses (SSTR2) [6].

Previously, SSTRs have been detected in human lymphoid tissues, lymphoid cell-lines and peripheral blood cells. [7-14]. RT-PCR-based SSTR subtype analysis indicated that human peripheral blood B- and T-lymphocytes express only SSTR3, while monocytes, upon activation with LPS, express SSTR2 (Lichtenauer-Kaligis, submitted). SSTRs are also present on cells derived from several hematological malignancies, including malignant lymphomas [7,15], acute myeloid leukemias and lymphoblastic leukemias [16,17]. However, which receptor subtype(s) are expressed on these malignant cells has not been investigated.

We have recently shown that, of the 5 SSTR subtypes, only SSTR2 is expressed on a primitive subset of normal bone marrow cells ($CD34^+/CD117^+$) (Oomen, in press). Interestingly, SSTR2 expression is lost when cells acquire immunophenotypic markers of differentiation towards the lymphoid, myeloid and/or erythroid lineages. We also found that somatostatin and octreotide, a stable analogue of somatostatin, act as chemoattractants for human primitive hematopoietic ($CD34^+$) cells, including the clonogenic progenitors CFU-GM and BFU-E. This somatostatin-mediated mechanism may be of importance *in vivo*, in the homing of BM progenitors/stem cells to different organs or to different niches within these organs, during development and/or adult life.

In this study we analyzed the expression pattern of SSTR subtypes on acute myeloid leukemia (AML) cells and investigated whether somatostatin influences migration of AML cells. Our results show that, like normal human bone marrow (hBM) cells, AML blasts exclusively express SSTR2. However, in contrast to normal hBM, SSTR2 expression in AML is not always restricted to the $CD34^+$ cells. AML cells showed variable migration properties towards an octreotide gradient, which correlated with the expression levels of SSTR2 on the AML cells. These findings indicate that SST-mediated mechanisms may influence the migratory behavior of AML blasts, which might have consequences for compartmentalization and spreading of the leukemic cells *in vivo*.

Materials and Methods

Isolation of AML cells

Bone marrow cells were obtained from 16 patients with AML (five M1, two M2, three M4 and six M5) following informed consent. The marrow cells were collected in Hank's Balanced Salt Solution (HBSS) supplemented with heparin. Mononuclear, T lymphocyte-depleted and monocyte-depleted (only M1 and M2 AML samples) cells were collected and cryopreserved prior to use as described previously [18,19]. The AML samples used contained at least 95% blasts.

Reverse transcriptase polymerase chain reaction (RT-PCR)

To determine SSTR subtype and somatostatin expression, RT-PCR was performed as described [20]. In brief, polyA$^+$ mRNA was isolated from 10^6 cells per sample using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway). cDNA was synthesized using the polyA$^+$ mRNA captured on the Dynabeads Oligo $(dT)_{25}$. One-tenth of each cDNA sample immobilized on the paramagnetic beads was used per amplification. Oligonucleotide primers specific for the human somatostatin receptor subtypes SSTR1-5 (SSTR2 primers are specific for SSTR2A), somatostatin and hypoxanthine-guanine phosphoribosyl transferase (HPRT) are described in Table 1. PCR was carried out in a DNA thermal cycler with heated lid (Perkin Elmer, Nieuwerkerk a/d IJssel, The Netherlands). After an initial denaturation at 95°C for 5 min, samples were subjected to 33 (SSTR2) or 40 (all others) cycles of denaturation at 94°C for 1 min, annealing for 2 min at 59°C and extension for 1 min at 72°C. PCR products were analyzed by electrophoresis on 1.5-2% agarose gels stained with ethidium bromide.

human	Sequence (5'- 3') [a]	Position [b]	PCR product
HSSTR1-FW§	ATGGTGGCCCTCAAGGCCGG	754	318 bp
HSSTR1-RV#	CGCGGTGGCGTAATAGTCAA	1071	
hSSTR2-FW [c]	GCCAAGATGAAGACCATCAC	214	414 bp
hSSTR2-RV	GATGAACCCTGTGTACCAAGC	627	
hSSTR3-FW	CCAACGTCTACATCCTCAACC	236	314 bp
hSSTR3-RV	TCCCGAGAAGACCACCAC	549	
hSSTR4-FW	ATCTTCGCAGACACCAGACC	547	321 bp
hSSTR4-RV	ATCAAGGCTGGTCACGACGA	867	
hSSTR5-FW	CGTCTTCATCATCTACACGG	596	226 bp
hSSTR5-RV	CCGTCTTCATCATCTACACGG	819	
hSST-14-FW	GATGCTGTCCTGCCGCCTCCAG	-1	349 bp
hSST-14-RV	ACAGGATGTGAAAGTCTTCCA	348	
hhprt-FW	CAGGACTGAACGTCTTGCTC	132	413bp
hhprt-RV	CAAATCCAACAAAGTCTGGC	544	

Table 1. Primers used in RT-PCR analysis of SSTR subtype expression

[a]). The sequences of the primers for hSSTR1 and hSSTR4 are derived and adapted from Wulfsen *et al.* [28], for hSSTR5 from Kubota *et al.* [29]. All other primers were designed by use of the Primer3! software (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) and the appropriate GenBank entries.

[b]). The position is given of the 5' nucleotide of the primer relative to the first nucleotide of the coding region in the cDNA sequence.

[c]). The SSTR2 primers are specific for SSTR2A.

§ FW: forward primer.

RV: reverse primer.

Flow cytometric analysis of somatostatin binding to AML blasts
To determine somatostatin binding to AML blasts, cells were incubated with 50 nM of Fluo-somatostatin™ (Advanced Bioconcept, Montreal, Quebec, Canada) for 45 min at RT in the dark. Fluo-SST binds to SSTR subtypes 1, 2 and 3 but not to SSTR4 and SSTR5 (according to the manufacturer). Non-specific binding was assessed in parallel incubations, in which a 100-fold excess of (D-Trp^8)-somatostatin-14 (BACHEM AG, Switzerland) was added. Human AML cells were double stained with R-phycoerythrin (PE) conjugated antibodies against human CD34 or CD117 (CD34-PE, CD117-PE,1:10 vol/vol; Beckman Coulter. Inc) or IgG_1-PE (1:10 vol/vol, Beckton Dickinson) for 30 min at 4 °C. Stained cells were suspended in phosphate buffered saline (PBS), supplemented with 0.05% NaN_3 and 1% fetal calf serum (FCS). Cells were then subjected to flow cytometric analysis on a FACScan (Becton-Dickinson, Sunnyvale, CA). A gate was set on the basis of forward sideward light scatter to exclude dead cells from the analysis.

Immunochemical detection of SSTR2
AML cytospins were stained with SSTR2A (R2-88) antibody as described earlier [20]. A standard streptavidin-biotinylated-peroxidase complex (ABC) kit (Biogenix, San Ramon, CA) was used to visualize the bound antibodies. Stainings with R2-88 antibody pre-adsorbed with the immunizing receptor peptides (at a concentration of 0.3 µg/ml = 100 nM) were performed as negative controls.

Cell migration assays
Transwell migration assays were performed with 5 µm pore filters (Transwell, 6.5 mm diameter, 24-well cell clusters; Costar, Cambridge, MA), pre-coated overnight at 4 °C with 20 µg/ml fibronectin (Bovine plasma: 0.1% in 0.5M NaCl/ 0.05 M Tris pH 7.5, Sigma F 1141) as previously described [21]. In brief, 2 x 10^5 cells in 100 µl migration buffer (Iscove's medium, 0.5% BSA) were added to the upper chamber and 0.6 ml of migration buffer was added to the lower chamber. Increasing concentrations of octreotide were added to the migration buffer in the lower chamber to determine the optimal concentrations required for migration. In some experiments, octreotide (10^{-10} M) was placed in the upper and/or lower chamber. Chambers were incubated at 37°C, 5% CO_2 for 4 hours. Cells migrating towards the lower chamber were counted using a cell counter (CASY®1/TTC, Schärfe Systems, Germany).

Statistical analysis
Standard linear regression analysis (SSTR2 mean fluorescence *versus* migration) was determined using a two tailed Spearman's Rho test. The correlation is considered significant at the 0.01 level (two-tailed).

Results

SSTR expression on human AML cells

Somatostatin receptors have been described to be present on human AML cells [16]. To determine which SSTR subtypes are expressed on these cells, we isolated mRNA from AML cells and performed RT-PCR analysis using primers specific for the different SSTR subtypes. Similar to normal human bone marrow, only SSTR2 transcripts were detected (Fig.1). We also investigated whether AML blasts produce somatostatin. In the 16 AML samples analyzed by RT-PCR, somatostatin transcripts could not be detected (Fig. 1), ruling out autocrine and/or paracrine effects on SSTR2-mediated migration. SSTR2 mRNA was found in all AML samples, indicating that at least basal levels of SSTR2 were produced. Flow cytometric analysis with Fluo-SS showed that AML cells express variable amounts of SSTR2 on the cell membrane (Fig. 2). Of the 16 cases of AMLs tested, 13 showed specific Fluo-SST binding, with a mean fluorescence intensity ranging from 1.2 to 17 (Table 2). In this cohort of AMLs no relationship was evident between morphological classification (French-American-British group) and the levels of Fluo-SST binding. As expected, Fluo-SST binding of AML cells could be completely blocked by not only D-Trp^8-SST, but also by octreotide, confirming that the binding capacities of these ligands for SSTR2 are similar(data not shown). The expression of SSTR subtype 2 was confirmed by immunochemistry using an antibody (R2-88) specific for SSTR2 (Fig. 3).

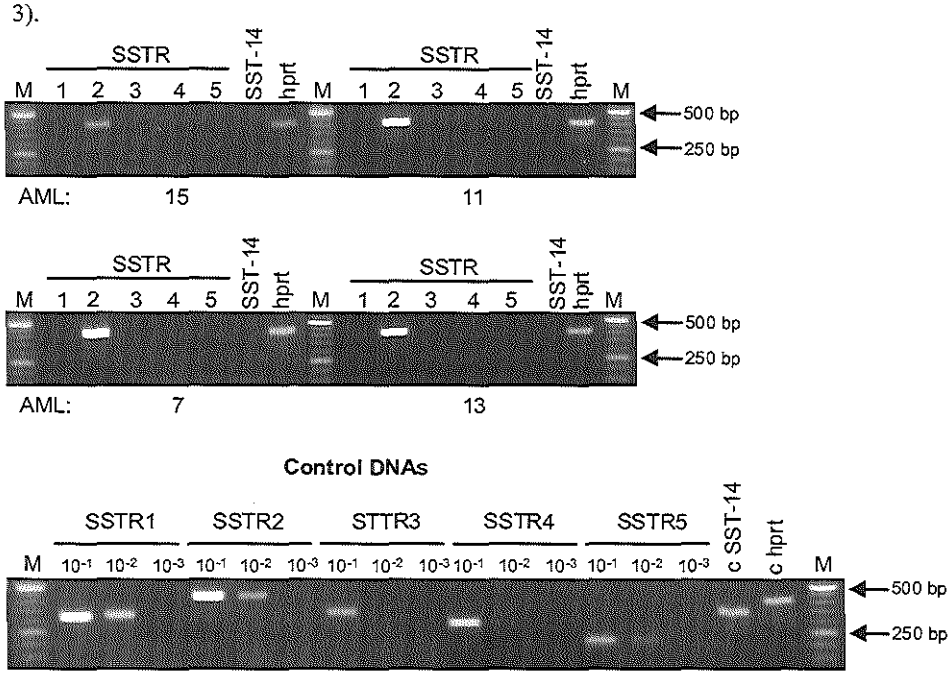

Figure 1. RT-PCR analysis of somatostatin receptor (SSTR) and somatostatin (SST-14) expression in AML samples. PCR with the hprt specific primers served as a positive control for the quality of the cDNA. Control DNA samples contained either cDNA (lanes c) from a control cell line known to express SST-14 and hprt mRNA, or different amounts of genomic DNA (lanes 10^{-1}, 10^{-2} and 10^{-3} µg). Mock-reverse-transcribed samples and controls with no template added did not yield PCR products (not shown). Marker lanes (M) contain a 50 bp DNA ladder.

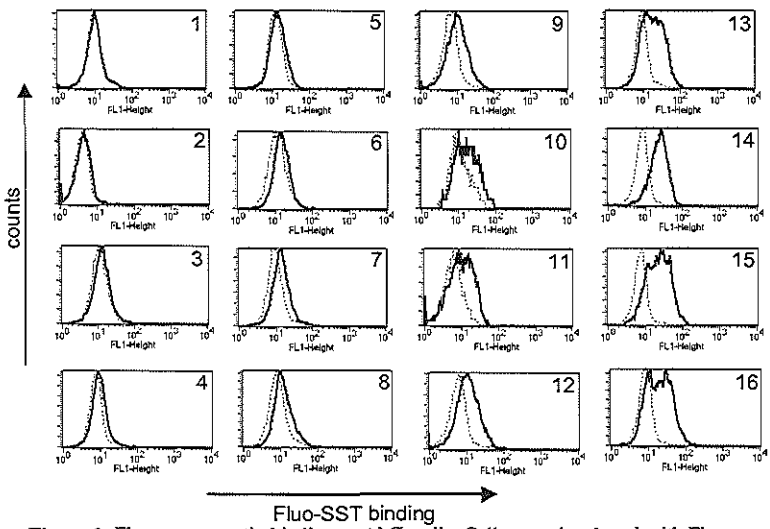

Figure 2. Fluo-somatostatin binding to AML cells. Cells were incubated with Fluo-somatostatin in the absence (solid line) or presence (broken line) of 100-fold molar excess of unlabeled somatostatin and analyzed by FACS.

		Fluo-SST binding			migration			Optimal oct
Pat. No.	FAB	(MF)	CD34	CD117	spontaneous	induced	fold induction	Concentration
1	M5b	0	+	+	960	1035	1.078	10^{-8}
2	M1	0.06	+	+	690	840	1.2	10^{-10}
3	M2	0.83	+	+	13800	16400	1.18	10^{-8}
4	M1	1.06	+	+	4545	5745	1.26	10^{-9}
5	M2	1.88	+	+	915	1950	2.13	10^{-8}
6	M4	2.45	+	+	4230	4672	1.1	10^{-10}
7	M1	3.27	+	+	15500	20000	1.29	10^{-11}
8	M5b	3.4	+	+	1245	2095	1.68	10^{-11}
9	M4	3.97	+	+	5550	8535	1.53	10^{-10}
10	M5a	4.01	-	+	1485	1868	1.25	10^{-10}
11	M1	4.67	-	+	2790	4478	1.61	10^{-10}
12	M4	5.73	+	+	5430	10650	1.96	10^{-11}
13	M5	10.93	-	+	787	6675	8.48	10^{-11}
14	M5a	14.93	+	+	7251	16600	2.3	10^{-10}
15	M5b	15.1	+	+	2085	7688	3.68	10^{-11}
16	M1	17.33	+	+	4815	10300	2.13	10^{-11}

Table 2. Fluo-SST binding and octreotide-induced migration in AML.
FAB: French-American-British AML classification.
MF: mean fluorescence after subtracting the MF of control samples (fluorescent signal with excess of unlabeled somatostatin)
+: expression of CD34 or CD117 on the AML blasts.
-: no expression of CD34 or CD117 on the AML blasts.
Spontaneous migration: control medium in the lower chamber.
Induced migration: octreotide-containing medium in the lower chamber.
Migration fold induction: data are expressed as fold induction of migration as compared to when no octreotide was added to the lower chamber (=1).
Optimal oct concentration: octreotide concentration (M) at which maximum cell migration occurred.

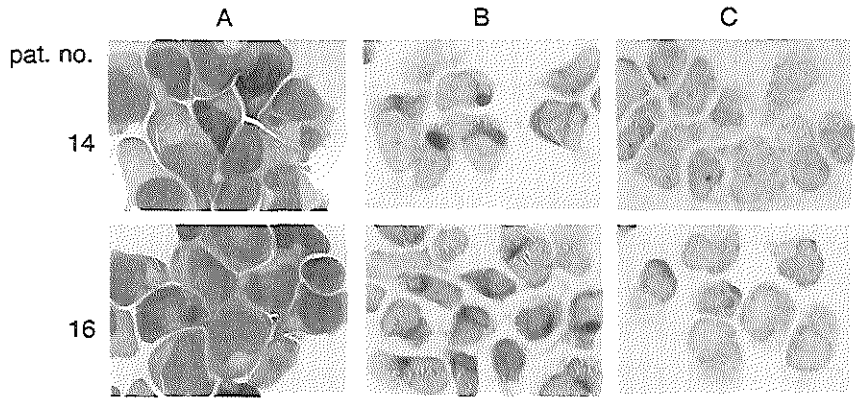

Figure 3. Immunochemical detection of SSTR2 (R2-88 antibody) in 2 AML samples. (A): hematoxylin stainings; (B) Immunoperoxidase staining with R2-88 and (C) Immunoperoxidase staining with R2-88 pre-adsorbed with 100 nM of immunizing peptide.

Distribution of SSTR among immunophenotypic subsets of AML

We have recently shown that SSTR2 expression in normal hBM is confined to $CD34^+$ and $CD117^+$ (c-kit^+) expressing cells (Oomen, submitted for publication). Strikingly, this restricted expression of SSTR2 was not seen in AML. In some cases, both $CD34^+/CD117^+$ and $CD34^-/CD117^-$ were found to express high levels of SSTR2 (Fig 4A), while in others $CD117^+$ AML cells showed a higher SSTR2 expression than $CD117^-$ cells (Fig 4B). Notably, also in a case of $CD34^-$ AML, high SSTR2 levels were found (Fig 4C). A summary of the Fluo-SST binding data is given in Table 2. These findings establish that AML blasts show a markedly heterogeneous SSTR2 expression, which frequently deviates from normal hBM progenitors.

Octreotide induces migration of human AML bone marrow cells in vitro

We then assessed the migration properties of AML cells towards an octreotide gradient in a transwell assay. The response of AML blasts from different patients to an octreotide gradient was heterogeneous (Fig. 5A). Octreotide-induced migration was dose-dependent, with maximal migration at concentrations between 10^{-11} M and 10^{-10} M in most cases. Migration towards a negative gradient (Oct in the upper chamber) was negligible (Fig. 5B). However, in certain cases of AML , octreotide induced migration in absence of a chemoattractant gradient (Oct in the upper and lower chambers) , which is indicative of chemokinesis, rather than chemotaxis (no. 14, Fig. 5B). Because AML cells from different patients showed variable spontaneous migration, we expressed the migration as fold induction of spontaneous migration (Table 2), which ranged from 1.1 (little or no induction) to 8.5. No relation was detected between spontaneous migration and SSTR expression. In contrast, octreotide-induced migration correlated with expression levels of SSTR2 on the cell surface (Fig. 6).

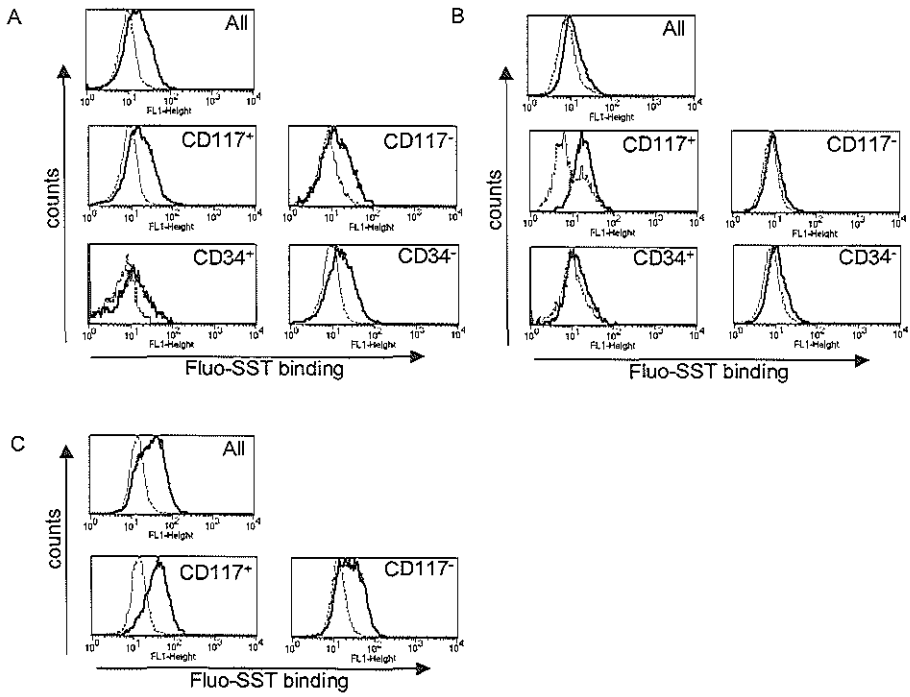

Figure 4. Fluo-somatostatin binding in subpopulations of AML cells. Cells were incubated with Fluo-somatostatin and subsequently with PE-conjugated antibody against human CD34 or PE-conjugated antibody against human CD117 (c-kit). Windows were set to restrict the analysis to CD34 and CD117 positive or negative cells. Cells were treated with Fluo-somatostatin in the absence (solid line) or presence (broken line) of 100-fold molar excess of unlabeled somatostatin. (A) AML#9. (B) AML#8. (C) AML#13: SSTR2 expression on CD34 negative AML.

Discussion

During different stages of fetal development and adult life primitive hematopoietic cells migrate between organs, e.g. fetal liver, bone marrow and spleen. In addition, stem cells can migrate towards the peripheral blood in response to several mobilizing agents. How the migratory behavior of hematopoietic stem cells and progenitor cells is governed is still largely unknown. Physiological stimuli of stem cell migration include various cytokines and chemokines. The major chemokine implicated in hematopoietic stem cell trafficking is stromal cell-derived factor-1 (SDF-1) [22]. The receptor for SDF-1, CXCR4, is a 7-transmembrane $G_{\alpha i}$-coupled receptor that belongs to the family of chemokine receptors. A recent study showed that CXCR4 is also expressed on primary AML leukemic cells in variable amounts [23]. The expression level of CXCR4 determined the migratory response to SDF-1, indicating that the receptor was functionally active in these cells.

We have recently demonstrated that SSTR subtype 2 is expressed on a primitive subset of normal human bone marrow cells ($CD34^+/CD117^+$) and that somatostatin induces migration of these cells in a transwell system (Oomen, in press). In the present study we analyzed the expression and function of SSTR2 in primary human

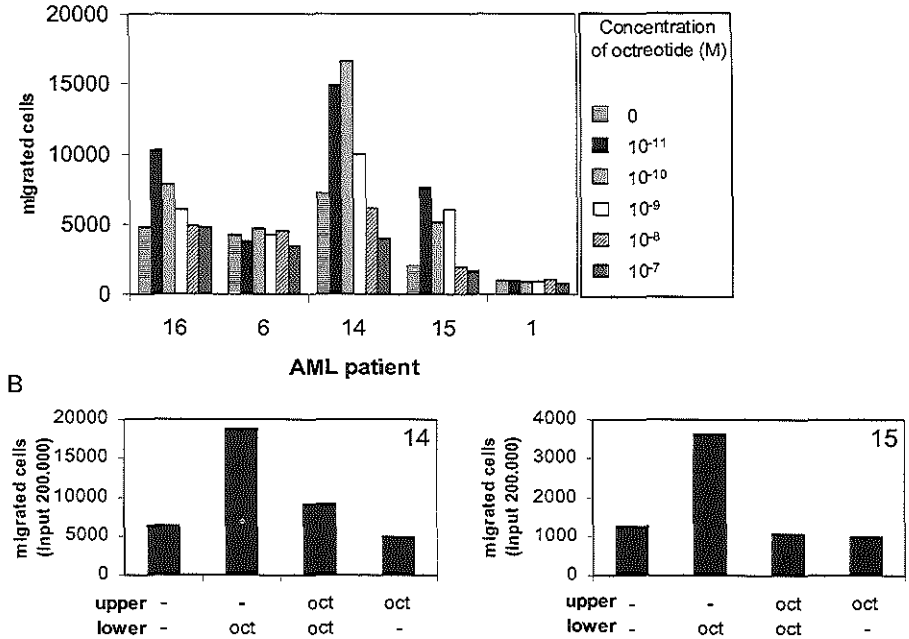

Figure 5. Octreotide-induced migration of AML cells. (A) Migration assay of AML cells in response to increasing concentrations of octreotide. A total of 200.000 cells were added to the upper chamber of a transwell system. After 4 hours, migrated cells recovered from the lower chamber were counted. Five representative cases are shown with a significant migratory response to octreotide (cases 14, 15 and 16) or without a response (cases 1 and 6). (B) Chemotactic versus chemokinetic activity of octreotide (oct) towards AML cells (AML#14 and AML#15). Oct (10^{-10} M) was added to upper and/or lower chambers as indicated. Data are expressed as the total amount of cells that migrated at each concentration.

leukemia cells of 16 patients. Expression of SSTR2 mRNA was detected in all AML samples, despite the fact that some of these samples were negative in Fluo-SST binding assays. Clearly, SSTR2 expression on AML cells is heterogeneous and, unlike normal hematopoietic precursors, not limited to CD34 and CD117 expressing cells. It is conceivable that receptors do not function properly in malignant cells, even when expressed in large amounts on the cell surface. However, our results show that the level of SSTR2 expression is directly related to the ability of the leukemic cells to migrate in response to the somatostatin analogue octreotide.

The presence of SSTR on a variety of human tumors suggests a role for somatostatin in the control of tumor development. Several tumors coexpress somatostatin along with SSTRs. The presence of SSTR in most of the neuroendocrine tumors together with the ability of many of those tumors to synthesize somatostatin point toward an autocrine regulatory feedback mechanism in these tissues [24]. However, we did not detect somatostatin transcripts in AML blasts by RT-PCR, which argues against such a mechanism in AML

Somatostatin receives major attention in the treatment of hypersecretive neuroendocrine tumors, because of its inhibitory action on hormone production, as

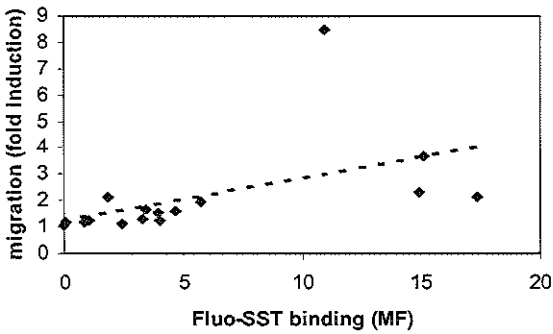

Figure 6. Correlation of SSTR2 expression and octreotide-induced migration of AML cells. A positive correlation (r = 0.799) was found between the expression level of SSTR2 (MF= mean specific fluorescence of Fluo-somatostatin) and migration (fold induction) in response to octreotide. The correlation was significant ($p<0.001$) as determined by a Spearman's Rho test.

well as its direct suppression of neoplastic cell growth [25]. This has also stimulated an interest in the development of somatostatin analogues, which lack systemic side effects, are relatively resistant to enzymatic degradation and thus show a longer half-life *in vivo* [26]. Somatostatin may act as a negative regulator of the proliferative activity of human myeloid leukemia [16] and preliminary data demonstrated a partial remission in 36% of patients with low-grade non-Hodgkin's lymphomas treated with octreotide [27]. The accurate evaluation of SSTR subtype status in hematological diseases might become of value in the treatment of these patients when new subtype selective analogues will be available for clinical applications like radiotherapy with radionuclide-coupled somatostatin analogues or chemotherapy with "toxin" coupled somatostatin analogues. Our finding that somatostatin and octreotide influence the migratory behavior of AML blasts, suggests that treatment with these peptides may also have consequences for compartmentalization and spreading of the leukemic cells *in vivo*. A future goal is to explore this in a relevant model, e.g. in NOD/SCID mice xenografted with human AML cells.

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Chapter 4.1

Somatostatin modulates G-CSF-induced but not interleukin-3-induced proliferative responses in myeloid 32D cells via activation of somatostatin receptor subtype 2

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The Hematology Journal (2001) **2** 322-329

Abstract

Introduction. Somatostatin, originally identified as a peptide involved in neurotransmission, functions as an inhibitor of multiple cellular responses, including hormonal secretion and proliferation. Somatostatin acts through activation of G-protein-coupled receptors of which 5 subtypes have been identified. We have recently established that human CD34/c-kit expressing hematopoietic progenitors and acute myeloid leukemia (AML) cells exclusively express SSTR2. A major mechanism implicated in the antiproliferative action of somatostatin involves activation of the SH2 domain-containing protein tyrosine phosphatase SHP-1. While 0.1-1 x 10^{-9} M of somatostatin, or its synthetic stable analog octreotide, can inhibit G-CSF-induced proliferation of AML cells, little or no effects are seen on GM-CSF- or IL-3-induced responses.

Materials and methods. To study the mechanisms underlying the antiproliferative responses of myeloblasts to somatostatin, clones of the IL-3-dependent murine cell line 32D that stably express SSTR2 and G-CSF receptors were generated.

Results. Similar to AML cells, octreotide inhibited G-CSF-induced but not IL-3-induced proliferative responses of 32D[G-CSF-R/SSTR2] cells. Somatostatin induced SHP-1 activity and inhibited G-CSF-induced, but not IL-3-induced, activation of the signal transducer and activator of transcription proteins STAT3 and STAT5.

Conclusion. Based on these data and previous results, we propose a model in which recruitment and activation of the tyrosine phosphatase SHP-1 by SSTR2 is involved in the selective negative action of somatostatin on G-CSF-R signaling.

Introduction

The peptide somatostatin (SST) exists in two biologically active forms (SST-14 and SST-28), which exert a range of biological functions, including inhibition of secretory and proliferative processes in many target organs [1]. The diverse effects of SST are mediated via high affinity G-protein-coupled membrane receptors. Somatostatin receptors (SSTR) are encoded by a family of five genes localized on different chromosomes [2]. The individual subtypes show a high degree of structural conservation across species. Somatostatin receptor subtype 2 (SSTR2) is expressed specifically on a primitive subset ($CD34^+$, $ckit^+$) of human and mouse bone marrow cells (SPMA Oomen, in press) and human acute myeloid leukemia (AML) cells also exclusively express SSTR2 [3].

Somatostatin and its stable analogs inhibit *in vitro* proliferation of various normal and malignant cell types, including mammary, prostatic, gastric, pancreatic, colorectal, and small cell lung cancer cells [4,5]. In addition to AML, hematological malignancies such as lymphoma and myeloma also express SSTR [6-9]. Antiproliferative effects of somatostatin on the proliferation of AML cells have been reported and these effects are strongest when proliferation is induced with granulocyte colony-stimulating factor (G-CSF). In contrast, SST inhibits interleukin-3- or GM-CSF-induced proliferative responses less frequently and less efficiently [7].

Both cytostatic and cytotoxic actions of SST have been described [10] and four of the SSTR subtypes (SSTR1-4) have been shown to stimulate protein tyrosine phosphatases (PTP) [10-16]. Therefore, major attention has focussed on the role of PTPs in the negative control of cellular responses by somatostatin. Activated SSTR2 forms a complex with the Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase-1 (SHP-1) [10-12,17], previously referred to as hematopoietic cell phosphatase, that is mainly expressed in hematopoietic cells [18-22]. Via its SH2 domains, SHP-1 also binds directly to certain tyrosine-phosphorylated cytokine receptors, including the erythropoietin receptor (EpoR), interleukin-2 receptor (IL-2R) and IL-3 receptor (IL-3R). Docking of SHP-1 to these receptors results in the down-modulation of tyrosine phosphorylation of the receptors, presumably due to inactivation of the receptor associated Jak kinases [23-28]. However, SHP-1 has also been reported to directly inhibit the activity of Jak kinases [29,30]. Finally, it has been suggested that SHP-1 can bind to and dephosphorylate the signal transducer and activator of transcription (STAT) protein STAT5, which is a major substrate of Jak kinases [31].

The receptor for G-CSF (G-CSF-R) is a member of the hematopoietin receptor superfamily, which forms homo-oligomeric complexes upon ligand binding [32]. Like other members of this receptor family, G-CSF-R lacks intrinsic tyrosine kinase activity but activates multiple cytoplasmic tyrosine kinases associated with these receptors. Major signaling substrates downstream of G-CSF-R are the Jak kinases Jak1, Jak2 and Tyk2 and the STAT proteins STAT3 and STAT5 [33-41]. Murine myeloid 32D cells have provided a useful model for studying the role of various signaling pathways activated by G-CSF-R in myelopoiesis [42,43]. SHP-1 protein levels are upregulated by G-CSF and SHP-1 inhibits G-CSF-induced proliferative responses in these cells [44].

To characterize the inhibitory action of SST on G-CSF responses of myeloblasts, SSTR2 and G-CSF-R were co-expressed in 32D cells. The stable SST analog octreotide (Oct) induced SHP-1 activity and inhibited G-CSF-induced proliferation and activation of STAT3 and STAT5 in 32D[G-CSF-R/SSTR2] cells. This suggests that activation of SSTR2 by Oct interferes with G-CSF signaling immediately downstream of G-CSF-R. In contrast, STAT5

activation by IL-3 was unaffected. These findings are discussed in view of a model in which SST transmodulates G-CSF-induced proliferative responses via recruitment and activation of SHP-1.

Materials and Methods

Construction of expression vectors
The retroviral vector pLNCX containing full length human G-CSF-R cDNA, used to generate 32D[G-CSF-R] cells, has been described previously [45]. For expression of SSTR2 in these cells, human SSTR2 cDNA in pBluescript (pBS) (a kind gift from GI Bell Howard Hughes Medical Institute, Chicago IL, USA) was excised from pBS and inserted into the *Bam*HI/*Sal*I site of the retroviral expression vector pBABE [46].

Cells and transfections
The IL-3-dependent murine myeloid cell line 32D[G-CSF-R] 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. These cells were electroporated with 10 µg *Pvu*I-digested pBABE[SSTR2] or pBABE empty vector control plasmid, using a Progenitor apparatus set at 230 V, 100 µF, and 1 sec. After 48 hours of incubation, cells were selected with puromycin at a concentration of 1 µg/ml. Multiple clones were expanded for further analysis. To determine SSTR2 expression levels, cells were incubated at room temperature (RT) for 60 min with 50 nM Fluo-somatostatin (Advanced Bioconcept, Montreal, Quebec, Canada). Non-specific binding was assessed by including 100-fold excess of unlabelled SST in parallel incubations. Samples were analysed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA, USA). Several independently derived cell lines were selected on the basis of homogeneous receptor expression.

Tritiated thymidine (^{3}H-TdR) uptake assay
DNA synthesis was determined by ^{3}H-TdR uptake as previously described [45]. Briefly, cells (0.2×10^6/ml) were incubated in triplicate in 100 µl serum free RPMI 1640 with titrated concentrations of recombinant human G-CSF (rhG-CSF, Amgen Incorporated, Thousand Oaks, WA, USA) or recombinant murine IL-3 (mIL-3) in 96-well plates. The SST analog, Oct (Novartis, Basel, Switzerland), was added to the cultures at final concentrations ranging from 10^{-6} M - 10^{-11} M and cells were cultured for 56 h. During the final 8 h of culture 0.1 µCi ^{3}H-TdR (2Ci/mmol/l; Amersham International, Amersham, UK) was added to each well. Cells were then harvested and ^{3}H-TdR incorporation was measured by liquid scintillation counting.

Preparation of nuclear extracts
Cells (2×10^6 per ml) were deprived of serum and factors by washing in RPMI 1640, followed by 4 h of incubation at 37°C in RPMI 1640. Cells were incubated with different concentrations of Oct and subsequently cultured in RPMI 1640 alone or in the presence of 100 ng/ml hG-CSF or 1 µg/ml mIL-3. At different time points, 10 volumes of ice-cold phosphate-buffered saline (PBS) supplemented with 10 µM Na_3VO_4 were added. Subsequently, cells were spun down and resuspended in ice-cold hypotonic buffer (20 mM HEPES pH 7.8, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2% Tween-20, 0.125 µM okadaic acid, 1 mM Pefabloc SC, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 50 µg/ml bacitracin and 50 µg/ml iodoacetamide) [47]. After vortexing for 10 sec, the nuclei were precipitated by centrifugation at 15000 *g* for 30 sec. Nuclear

extracts were prepared by resuspension of the nuclei in high-salt buffer (hypotonic buffer with 420 mM NaCl and 20% glycerol) and extraction of proteins by rocking for 30 min at 4°C. Insoluble materials were removed by centrifugation at 4°C for 15 min at 15000 *g*.

Electrophoretic mobility shift assay (EMSA)
Nuclear extracts from 1×10^6 cells were incubated for 20 min at RT with 0.2 ng of ^{32}P-labeled double-stranded oligonucleotide (5-10 $\times$ 10^3 c.p.m.) and 2 µg of poly(dI-dC) in 20 µl of binding buffer (13 mM HEPES, pH 7.8, 80 mM NaCl, 3 mM NaF, 3 mM $NaMoO_4$, 1 mM DTT, 0.15 mM EDTA, 0.15 mM EGTA and 8% glycerol) [48]. The following oligonucleotides were used: 5-AATTTCCTGTAACAT, the STAT5 binding site of the bovine β-casein promoter [49] and m67 (5-CATTTCCCGTAAATC) a mutated STAT3 binding site derived from the sis-inducible element (SIE) of the human c-fos gene [50]. DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gels containing 5% glycerol in 0.25 $\times$ TBE. The gels were dried and subsequently analyzed by autoradiography. Equal loading was verified on the basis of the signal intensity of radioactive free probe.

Protein tyrosine phosphatase (PTPase) activity assay
Cells (1×10^6 /ml) were deprived of serum and factors by culture in RPMI 1640 medium for 4 h at 37°C. Cells were then incubated with either RPMI 1640 medium alone or with medium containing 5 x 10^{-7} µM Oct. At different time points, 10 volumes of ice-cold PBS were added. Subsequently, cells were pelleted and lysed by incubation for 30 min at 4°C in lysis buffer (20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% Nonidet P-40, 10% glycerol, 2 mM Na_3VO_4, 1 mM Pefabloc SC, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 50 µg/ml bacitracin and 50 µg/ml iodoacetamide). Insoluble materials were removed by centrifugation (15 min, 15000 *g*) at 4°C. Immunoprecipitations were performed on the cleared cell lysates by incubation for 3 h at 4°C with anti-SHP-1 antibody (No. P17320, Transduction Laboratories, Lexington, KY, USA). Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) were then added for 1 h at 4°C. Subsequently, the loaded beads were washed four times with ice-cold lysis buffer, once with PTPase assay buffer (25 mM sodium acetate, pH 5.0, 2 mM dithiothreitol, 20% v/v glycerol) and resuspended in a final volume of 50 µl PTPase assay buffer. The phosphatase assay was started by the addition of sodium *p*-nitrophenyl phosphate to a final concentration of 5 mM. After incubation at 25°C for 20 min, the reaction was terminated by the addition of 0.2 mM NaOH, and the A_{405nm} of the supernatant was measured [51]. The pellets were analysed by SDS-PAGE and Western Blot analysis with anti-SHP-1 to confirm that equal amounts of SHP-1 were immunoprecipitated.

Statistical analysis
The results are expressed as the mean ± standard deviation (SD) of data obtained from at least three different experiments. The data were analysed by Student's *t* test and differences were considered significant at p values < 0.05.

Results

Binding of somatostatin to 32D[G-CSF-R/SSTR2] cells
Expression levels of SSTR2 were determined using Fluo-somatostatin in FACS analysis. Cell clones expressing significant levels of somatostatin binding sites were selected for further analysis. An example of such a clone (32D[G-CSF-R/SSTR2]) is shown in Figure 1.

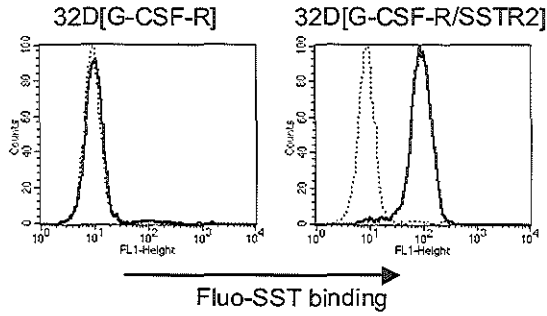

Figure 1. Expression of SSTR2 in 32D cells. FACS analysis of SSTR2 expression in 32D cells. The solid line shows the amount of fluorescence after labeling with Fluo-SST while the broken line represent the fluorescent signal remaining after displacement of Fluo-SST by unlabeled somatostatin.

Octreotide inhibits G-CSF-induced proliferation of 32D[G-CSF-R/SSTR2] cells

To assess the role of SSTR2-mediated signaling on myeloid cell proliferation, the effects of increasing concentrations of Oct on G-CSF- or IL-3-induced DNA synthesis were determined. Oct inhibited the G-CSF-induced proliferative responses of 32D[G-CSF-R/SSTR2] cells with a characteristic biphasic dose response relationship, showing maximal inhibition at a concentration of 10^{-9} M of Oct (Figure 2A). A narrower titration in the range of 10^{-10} M and 10^{-8} M of Oct again showed a bell-shaped dose response curve (Figure 2B). In contrast, IL-3-induced responses were not affected by Oct (Figure 2C).

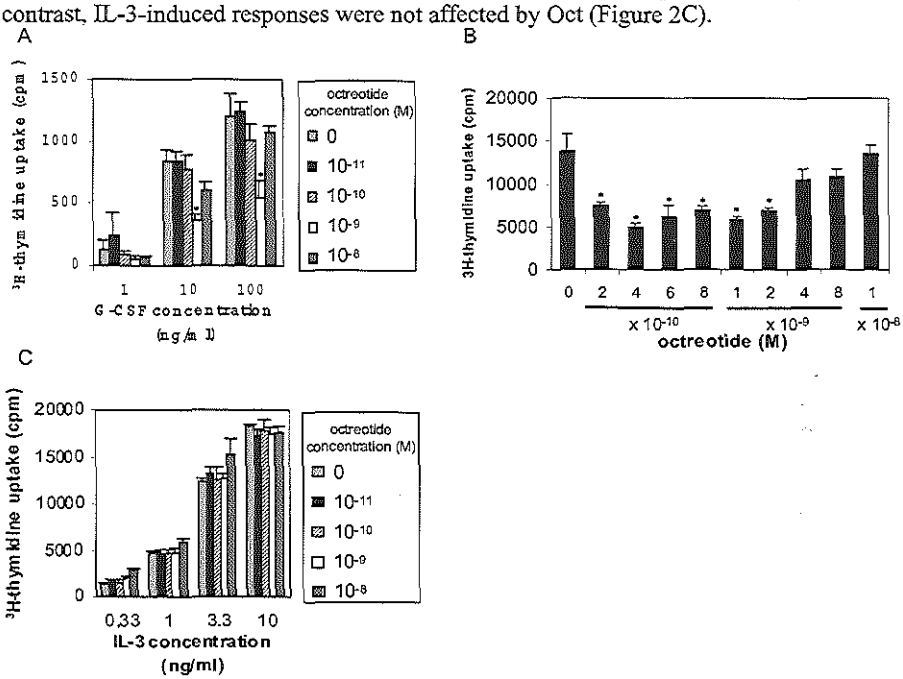

Figure 2. Effect of Oct on G-CSF- and IL-3-induced proliferation of 32D[G-CSF-R/SSTR2] cells. The proliferation responses of 32D[G-CSF-R/SSTR2] cells were evaluated in a ^{3}H-thymidine uptake assay, as described under Material and Methods. (A) Proliferation in response to different concentrations of G-CSF. (B)) Proliferation in response to 100 ng/ml G-CSF. (C) Proliferation in response to different concentrations of IL-3. The asterisk (*) indicates statistical significant inhibition compared with control (without Oct) at P values <0.05.

Octreotide induces SHP-1 activity in 32D[SSTR2] cells

Treatment of 32D[G-CSF-R/SSTR2] cells with Oct resulted in an approximately 2.5-fold increase in SHP-1 activity, which peaked between 1 and 10 min after stimulation, and returned to basal levels after 30 min. (Figure 3). No such Oct-induced increase in SHP-1 activity was seen in 32D control cells (data not shown).

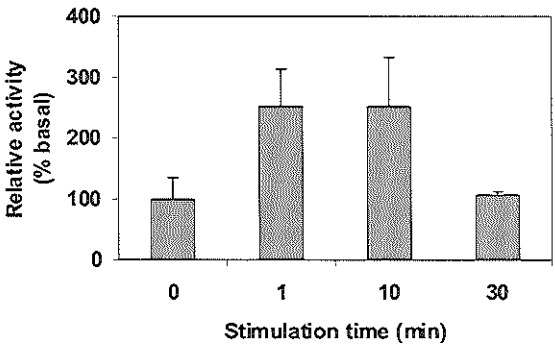

Figure 3. Induction of SHP-1 activity by Oct. 32D[G-CSF-R/SSTR2] cells were stimulated with Oct for the indicated times, and cell lysates analysed for tyrosine phosphatase activity following immunoprecipitation with anti-SHP-1. Values shown are the mean and standard error of three independent experiments, relative to the basal activity being set at 100% (A_{405} = 0.207).

Octreotide specifically inhibits G-CSF-induced STAT activation

Because Jak kinases have been identified as major substrates for SHP-1, we postulated that Oct-induced SHP-1 activity might have a negative effect on G-CSF-induced Jak activation. However, a negative effect of Oct on G-CSF-induced Jak activation by performing anti-phosphotyrosine Western blots or kinase assays on Jak1 or Jak2 immunoprecipitates was not demonstrated. Nonetheless, because these assays are not very sensitive, the possibility that G-CSF-induced Jak kinase activity was reduced by Oct treatment cannot be excluded. On the other hand, significant inhibitory effects of Oct on G-CSF-induced activation of STAT3 and STAT5, as measured with EMSA, were noted, whereas the levels of IL-3-induced STAT5 activation remained constant (Figure 4A). The reduced levels of STAT5 activation correlated with Oct concentrations, with a maximal inhibition at 10^{-6} M. Essentially similar data were obtained for G-CSF-induced activation of STAT3 (Figure 4B). Although the magnitude of G-CSF-induced STAT5 activation was clearly reduced, the time kinetics of activation were not significantly influenced by Oct treatment (Fig. 4C).

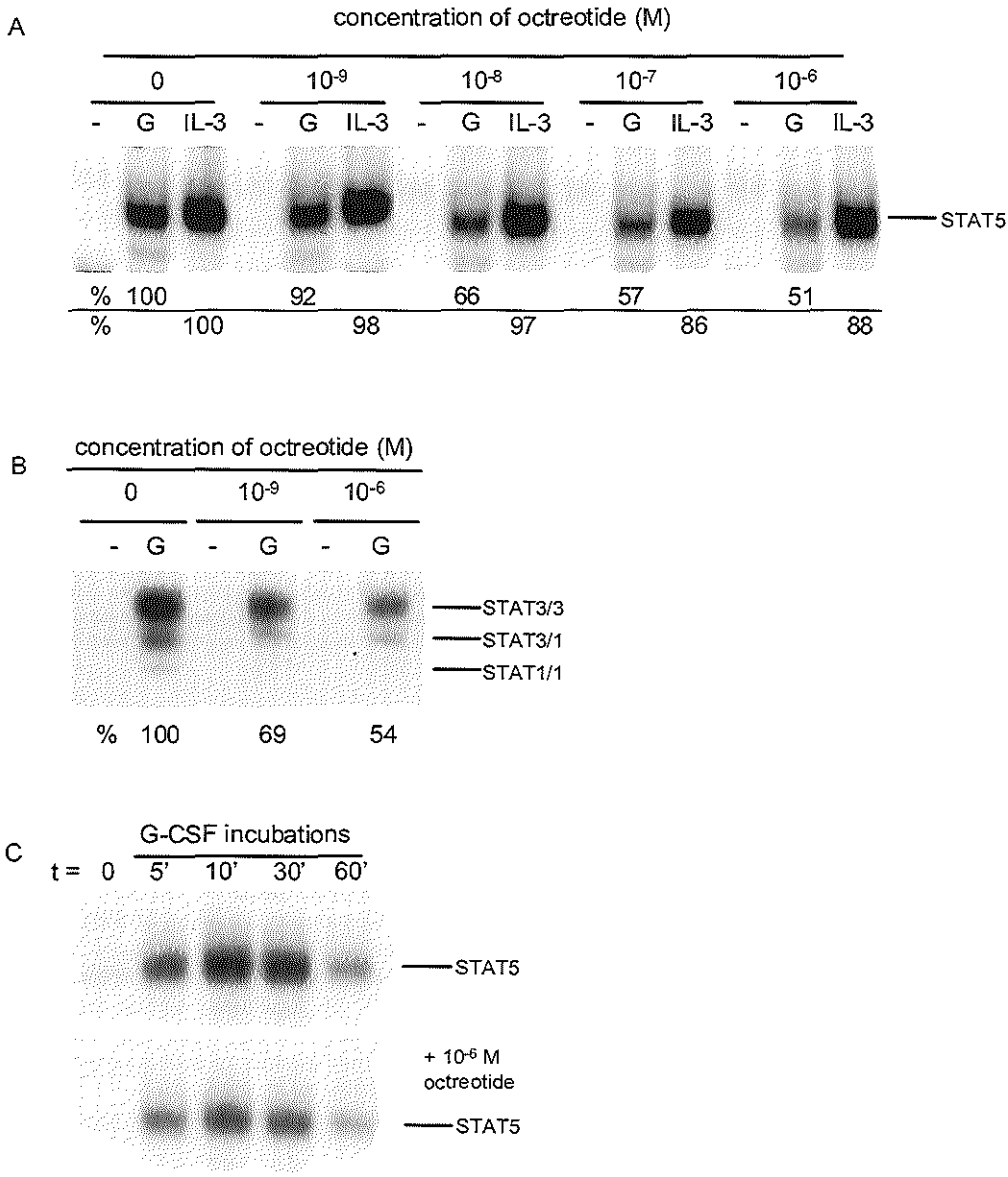

Figure 4. Effect of Oct on G-CSF-induced STAT5 and STAT3 activation. EMSA of nuclear extracts from 32D[G-CSF-R/SSTR2] cells. Nuclear extracts were prepared and incubated with ^{32}P-labeled double-stranded (A) β-cas or (B) m67 oligonucleotide probes as outlined in material and methods. After serum- and growth factor-deprivation and addition of different concentrations of Oct, cells were incubated for 10 minutes at 37 °C without factor (-), with 100 ng/ml G-CSF (G) or 1 μg/ml IL-3. (C) Time course of 100 ng/ml G-CSF-induced STAT5 activation. Cells are incubated and treated as under A.

Discussion

Previously, proliferation inhibitory effects of SST and Oct on primary AML cells were reported to be partial and dependent on the type of growth factor used to stimulate DNA synthesis [7]. Suppression of proliferation is efficient when AML cells are stimulated with G-CSF, and less so, or even absent, in the presence of IL-3 or GM-CSF [7]. The reason for this discrepancy remains unclear. AML cells from individual patients are heterogeneous in their *in vitro* behavior and proliferation is often influenced by complex autocrine regulatory loops [52]. These samples are therefore less suitable for dissecting more subtle mechanisms modulating cellular responses.

SSTR2 is the single SSTR subtype expressed on human bone marrow cells (SPMA Oomen, unpublished). SSTR2 expression is limited to the primitive, CD34 and c-kit-expressing subset of hematopoietic precursors and SSTR2 are present on human AML cells at variable densities [3]. To study the mechanisms underlying the growth inhibition action of somatostatin on AML cells, we generated a model based on the well characterized 32D system expressing both human G-CSF-R and SSTR2. Responses of 32D[G-CSF-R/SSTR2] to Oct are largely similar to those of human AML cells, which makes this a relevant model for studying SSTR2-controlled mechanisms of growth inhibition in myeloblasts. For instance, a similarity between Oct responses of primary AML cells and the 32D[G-CSF-R/SSTR2] cells is the bell-shaped dose effect relationship, which most likely reflects desensitization of receptors after prolonged activation of high somatostatin concentrations exposure [53]. This bell-shape response was not seen in the STAT DNA binding assays (EMSA). However, this was not unexpected, since these activities were measured 10 min after stimulation with G-CSF or IL-3 and Oct. In this time frame, effects of desensitization will not be measurable.

A number of studies have shown that SHP-1 acts as a negative regulator of hematopoiesis. Moreover, various hematopoietic abnormalities and hypersensitivity to growth factors have been demonstrated in mice carrying the mutation in the SHP-1 gene that results in moth eaten phenotype [54-56]. Viable moth eaten mice have increased myelopoiesis and show an accumulation of morphologically and phenotypically immature granulocytes, suggesting a role for SHP-1 in granulocytic differentiation. Importantly, *in vitro* binding reveals that the SH2 domains of SHP-1 are unable to bind directly to tyrosine-phosphorylated G-CSF-R [44], suggesting that SHP-1 modulates G-CSF-mediated responses in hematopoietic cells via indirect mechanisms. While SHP-1 is upregulated at the protein level by G-CSF in 32D cells and inhibits G-CSF-induced proliferation of these cells [44], expression studies in cell lines harboring different G-CSF-R forms and *in vitro* binding analysis indicated that SHP-1 does not dock to the activated G-CSF-R via its SH2 domains, as has been demonstrated for a varying of other cytokine receptors.

The model proposed, in which SHP-1 plays a central role in the negative effects of somatostatin on G-CSF signaling (Figure 5), shows that SHP-1 is expressed at increased levels following G-CSF induction [44] and is activated by somatostatin via SSTR2. Subsequently, SHP-1 down-modulates G-CSF-R-induced STAT activity and proliferation. Because SHP-1 does not bind directly to G-CSF-R, an alternative mechanism, the nature of which is unclear, must be involved. Although the effects of SHP-1 on cytokine receptor signaling have mainly been attributed to direct SH2 binding to tyrosine-phosphorylated receptors proteins [23-27], other studies have suggested that SHP-1 can also interact directly with other components of the receptor complex [29-31]. For instance, SHP-1 can associate directly with Jak2 through a new mechanism, that is independent of SH2 domain-

phosphotyrosine interaction [30]. This association leads to SHP-1 activation and dephosporylation of Jak2, and provides a potentially important mechanism for down-regulating the kinase phosphorylation without a requirement for SHP-1 binding to phosphotyrosine receptors. However, despite several efforts, we have been unable to coimmunoprecipitate Jak kinases with SHP-1 from 32D cell lysates. Although this might argue against a direct association between SHP-1 and Jaks, we do not exclude that the inability to coprecipitate Jaks with SHP-1 may be due to technical limitations of the immunoprecipitation assay, particularly in view of the relatively low abundance of Jak proteins in 32D cells. However, an alternative possibility is that SHP-1 affects other kinases, for example those of the Src family, which have also been implicated in the activation of STAT proteins [57] and G-CSF-induced proliferation [58].

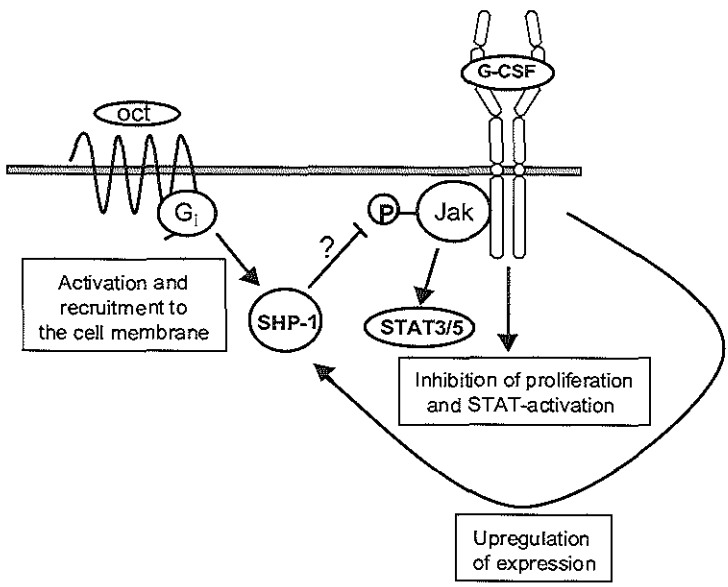

Figure 5. Hypothetical scheme of the recruitment of SHP-1 to the cell surface by SSTR2. Inhibition of G-CSF-induced proliferation via recruitment of SHP-1 by SSTR2.

In conclusion, we suggest that activation of SHP-1 by Oct is the initiating step for SSTR2-mediated inhibition of G-CSF-induced proliferative responses of myeloblasts. We hypothesize that this involves both activation and recruitment of SHP-1. We would argue that such an effect is limited or absent in IL-3-stimulated cells, because SHP-protein levels are not upregulated by IL-3 [44]. Moreover, the IL-3-R β-chain, itself, contains a SHP-1 docking site [24], which would make the recruitment of SHP-1 via an alternative mechanism redundant.

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Chapter 4.2

The SH2 domain-containing protein tyrosine phosphatase SHP-1 is induced by granulocyte colony-stimulating factor (G-CSF) and modulates signaling from the G-CSF receptor

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Leukemia (2000) 14 1284-1291

Abstract
The SH2 domain-containing protein tyrosine phosphatase SHP-1 is expressed widely in the hematopoietic system. SHP-1 has been shown to negatively control signal transduction from many cytokine receptors by direct docking to either the receptor itself, or to members of the Jak family of tyrosine kinases which are themselves part of the receptor complex. *Motheaten* and *viable motheaten* mice, which are deficient in SHP-1, have increased myelopoiesis and show an accumulation of morphologically and phenotypically immature granulocytes, suggesting a role for SHP-1 in granulocytic differentiation. Here we report that SHP-1 protein levels are up-regulated during the granulocyte colony-stimulating factor (G-CSF)-mediated granulocytic differentiation of myeloid 32D cells. Enforced expression of SHP-1 in these cells leads to decreased proliferation and enhanced differentiation, while introduction of a catalytically-inactive mutant produces increased proliferation and results in a delay of differentiation. *In vitro* binding revealed that the SH2 domains of SHP-1 are unable to associate directly with tyrosine-phosphorylated G-CSF receptor (G-CSF-R). Furthermore, over-expression of SHP-1 in Ba/F3 cells expressing a G-CSF-R mutant lacking all cytoplasmic tyrosines also inhibited proliferation. Together these data suggest that SHP-1 directly modulates G-CSF-mediated responses in hematopoietic cells via a mechanism that does not require docking to the activated G-CSF-R.

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a major regulator of neutrophilic granulocyte production [1-4]. Its actions are mediated via a specific cell-surface receptor, the G-CSF-R, which forms homo-oligomeric complexes upon ligand binding [5]. Like other members of the hematopoietin receptor superfamily, the G-CSF-R lacks intrinsic tyrosine kinase activity but activates numerous cytoplasmic tyrosine kinases and signaling cascades [2,5,6]. Important signaling molecules downstream of the G-CSF-R include certain members of the Janus tyrosine kinase (Jak) and signal transducer and activator of transcription (STAT) families of proteins [7-16], the Src kinases $p55^{Lyn}$ and $p56/59^{Hck}$ [17-19], and components of the $p21^{ras}$/Raf/MAPK pathway [11,20-23].

Src homology 2 (SH2) domain-containing protein-tyrosine phosphatase (PTPase), or SHP-1 (previously called HCP, SHP, PTP1C and SHPTP1), is a cytoplasmic protein, bearing tandem SH2 domains in the amino region, and a PTPase domain at its carboxyl terminus, predominantly expressed in hematopoietic cells [24-28]. *Motheaten* and *viable motheaten* mice have mutations in their SHP-1 gene and are characterized by increased myelopoiesis, as well as hypersensitivity to extracellular stimuli and heightened phosphorylation in their hematopoietic cells [29-33]. In addition, these mice show an accumulation of morphologically and phenotypically immature granulocytes [30,34,35]. However, whether these effects were due to aberrant G-CSF-R signaling remained unknown.

SHP-1 has been shown to bind directly to a number of tyrosine-phosphorylated receptors via its SH2 domains, including the erythropoietin receptor (EpoR), interleukin-2 receptor (IL-2R), interleukin-3 receptor (IL-3R), and the receptor for stem cell factor (c-Kit) [36-41]. Recruitment of SHP-1 in this manner facilitates the down-modulation of tyrosine phosphorylation and mitogenic signaling from the respective receptors [36,39-41]. However, other studies have shown that SHP-1 can also interact directly with members of the Jak kinase family, including Tyk2 and Jak2, to modulate both their tyrosine phosphorylation and activity [42,43], or to STAT proteins themselves [44].

Myeloid 32D cells have provided a useful model for studying the regulation of granulocytic development. Stimulation of these cells with G-CSF produces transient proliferation followed by differentiation into mature neutrophilic granulocytes in response to G-CSF [16,45]. In this study, we sought to identify key molecules that might participate in the switch from proliferation to differentiation in these cells. We show that SHP-1 is up-regulated following G-CSF stimulation, with kinetics suggestive of a role for this protein in the termination of the proliferative phase of these cells. However, enforced expression of wild-type and mutant SHP-1 in 32D cells revealed not only a negative role for SHP-1 in proliferation, but also a positive role in differentiation mediated by G-CSF. Finally, we show that SHP-1 impacts directly on G-CSF-R signaling, but that these effects are not mediated by direct docking to the activated receptor.

Materials and Methods

Cell culture
The subline of the IL-3-dependent murine myeloid cell line 32D.cl3, called 32D.cl8.6 [16], and the IL-3-dependent murine pro-B cell line, Ba/F3 [46] were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng/ml of murine IL-3, at 37°C and 5 % CO_2.

Plasmids and transfections
We have previously described the 32D.cl8.6 clone expressing the wild-type (WT) G-CSF-R [16] and the Ba/F3 clones expressing the WT and tyrosine-null mutant (mO) G-CSF-R [15]. Empty pBabe vector and pBabe expression constructs of wild-type and catalytically-inactive (C453S) SHP-1 (SHP-1^{wt} and SHP-1^{mut}, respectively) were linearized by *Pvu*I-digestion and transfected into these 32D and Ba/F3 clones by electroporation. After 48 or 24 hours of incubation, respectively, cells were selected with Puromycin (Gibco-BRL, Breda, The Netherlands) at concentrations of 1.0 and 1.25 µg/ml, respectively, with multiple clones expanded for further analysis.

Analysis of G-CSF-R expression
To check G-CSF-R expression levels, cells were incubated at room temperature for 60 min with 10 µg/ml of biotinylated mouse anti-human G-CSF-R monoclonal antibody LMM741 (PharMingen, San Diego, CA), then at 4°C for 60 min sequentially with 5 µg/ml of PE-conjugated streptavidin, 5 µg/ml of biotinylated anti-streptavidin antibody, and finally 2 µg/ml of PE-conjugated streptavidin, with washing between each antibody step. Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Only cell lines that had maintained homogeneous G-CSF-R expression were selected for further analysis.

Preparation of cell lysates and Western blotting
To obtain cell lysates, cells were pelletted, washed with ice-cold phosphate-buffered saline (PBS), and lysed by incubation for 30 min at 4°C in pTyr lysis buffer (1 % Triton X-100, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1 mM Na_3VO_4, 1 mM DTT, 1 mM Pefabloc SC, 50 µg/ml aprotinin, 50 µg/ml leupeptin, 50 µg/ml bacitracin) followed by centrifugation at 13 000 *g* for 15 min. The soluble proteins were mixed with sample buffer, separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto nitrocellulose (0.2 µm; Schleicher & Schuell, Dassel, Germany). Filters were blocked by incubation in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05 % (v/v) Tween-20) containing 0.6 % (w/v) BSA. Antibodies used for Western blotting were anti-SHP-1 (#P17320, Transduction Laboratories, Lexington, KY) and anti-SHP-2 (sc-280, Santa Cruz Biotechnology, Santa Cruz, CA), and were diluted in TBST containing 0.6 % (w/v) BSA. After washing with TBST, immune complexes were detected with horseradish peroxidase-conjugated species-specific antiserum (DAKO, Glostrup, Denmark), followed by enhanced chemiluminescence reaction (DuPont, Boston, MA). In some instances, membranes were stripped in 62.5 mM Tris-HCl pH 6.7, 2 % SDS and 100 mM ß-mercaptoethanol at 50 °C for 30 min, reblocked, washed, and reprobed.

Northern blot analysis
RNA was extracted from cells using Ultraspec™II (Biotecx Laboratories Inc., Houston, TX), separated on agarose-formaldehyde gels and transferred to Hybond N^+ filters (Amersham Life Sciences, Amersham, UK) according to the manufacturers' instructions. Probes were a 1 kb

*Bam*HI-*Bgl*II fragment from murine SHP-1 and an 0.8 kb PCR fragment of murine GAPDH which were ^{32}P-labeled by random priming (Boehringer, Mannheim, Germany).

Cell proliferation and morphological analysis

To determine proliferation, cells were incubated at an initial density of 1-2×10^5 cells/ml in 10% FCS/RPMI medium supplemented with 100 ng/ml of human G-CSF, 10 ng/ml of murine IL-3, or without growth factors. The medium was replenished every 1-2 days, and the cell densities were adjusted to 1-2×10^5 cells/ml. Viable cells were counted on the basis of trypan blue exclusion. To analyze the morphological features, cells were spun onto glass slides and examined after May-Grünwald-Giemsa staining.

Far Western analysis

The cytoplasmic domain of the human G-CSF-R (aa630-813), and the SH2 domains of SHP-1 (aa1-106; aa107-267) and SHP-2 (aa1-107; aa110-215), were cloned into pET-15b (Novagen, Madison, WI, USA) and pGEX-2TK (Amersham Pharmacia Biotech - Benelux, Roosendaal, The Netherlands) respectively, utilizing standard PCR protocols to amplify the appropriate coding regions. For the production of tyrosine-phosphorylated G-CSF-R cytoplasmic domain, the pET clone was introduced into the *E. coli* strain TKB1 (Stratagene, La Jolla, CA), which contains an inducible tyrosine kinase. Fusion protein was produced according to the manufacturer's instructions, and subsequently ^{32}P-labeled with heart muscle kinase. For production of GST and GST-SH2 fusions, plasmids were transformed into XL-1 Blue (Stratagene), with proteins expressed and purified on glutathione-Sepharose 4B beads as described [47]. The purified proteins were electrophoresed on 10 % SDS-PAGE gels [48], and electrophoretically transferred to Hybond-C membranes (Amersham Pharmacia Biotech). The membranes were then processed through a denaturation-renaturation cycle [49] and probed with the ^{32}P-labeled G-CSF-R, as described [19,50].

Electrophoretic mobility shift assay (EMSA)

Cells were deprived of serum and factors for 4 h at 37°C in RPMI 1640 medium at a density of 1 × 10^6 per ml, and then stimulated with either RPMI 1640 medium alone or in the presence of 100 ng/ml human G-CSF. At different time points, 10 volumes of ice-cold phosphate-buffered saline (PBS) supplemented with 10 μM Na_3VO_4 were added. Cells were then pelletted and resuspended in ice-cold hypotonic buffer (20 mM HEPES pH 7.8, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.2 % Tween-20, 0.125 μM okadaic acid, 1 mM Pefabloc SC, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml bacitracin and 50 μg/ml iodoacetamide) [51]. Cells were vortexed for 10 s and the nuclei pelletted by centrifugation at 15 000 *g* for 30 s. Nuclear extracts were prepared by resuspension of the nuclei in high-salt buffer (hypotonic buffer with 420 mM NaCl and 20 % glycerol) and extraction of proteins by rocking for 30 min at 4°C. Insoluble materials were removed by centrifugation at 4°C for 15 min at 15 000 *g*. Nuclear extracts of approximately 0.4 × 10^6 cells were incubated for 20 min at room temperature with 0.2 ng of ^{32}P-labeled double-stranded oligonucleotide (5-10 × 10^3 c.p.m.) and 2 μg of poly(dI-dC) in 20 μl of binding buffer (13 mM HEPES, pH 7.8, 80 mM NaCl, 3 mM NaF, 3 mM $NaMoO_4$, 1 mM DTT, 0.15 mM EDTA, 0.15 mM EGTA and 8 % glycerol) [52]. The oligonucleotide probes used in this study were m67 (5'-CATTTCCCGTAAATC), a high-affinity mutant of the sis-inducible element (SIE) of the human c-*fos* gene [53], which binds STAT1 and STAT3, and β-cas (5'-AGATTTCTAGGAATTCAATCC), derived from the 5' region of the β-casein gene [54], which binds STAT5 and STAT1. The DNA-protein complexes were separated by electrophoresis on 5 % polyacrylamide gels containing 5 % glycerol in 0.25 × TBE. The gels were dried and subsequently analyzed by autoradiography.

Results

SHP-1 is up-regulated during G-CSF-induced granulocytic differentiation

We have previously shown that 32D cells expressing the wild-type (WT) G-CSF-R (32D[WT] cells) proliferate transiently in response to G-CSF, followed by terminal granulocytic differentiation into mature neutrophils [16,45]. To explain the transition from the proliferative phase to the differentiation phase, we hypothesized that the levels of an important signaling molecule might be modulated by G-CSF treatment. Therefore, we examined the levels of a number of signaling molecules using Western blot analysis, including STAT3, STAT5, Shc, Grb2, PI3K, Hck and the tyrosine phosphatases SHP-1 and SHP-2. Of those proteins analyzed, only the SHP-1 protein showed increased levels in response to G-CSF-treatment (Figure 1A). As a control the blot was stripped and re-probed with SHP-2 to confirm equal loading. Interestingly, Northern blot analysis revealed no similar induction of SHP-1 at the mRNA level (Figure 1B), indicating that some form of post-transcriptional control is relieved by G-CSF treatment to account for the increase in SHP-1 protein levels.

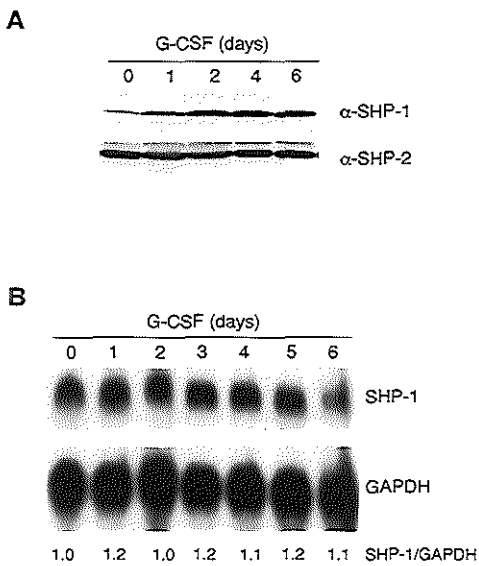

Figure 1. Induction of SHP-1 during G-CSF-induced differentiation of myeloid 32D cells.
(A) Western blot analysis of total lysates from 32D[WT] cells stimulated with G-CSF for the times shown. Duplicate filters were probed with antiserum to either SHP-1 or SHP-2, as indicated.
(B) Northern blot analysis of total RNA extracted from 32D[WT] cells stimulated with G-CSF for the times shown. The filter was hybridized sequentially with probes to murine SHP-1 followed by murine GAPDH, as indicated. The ratio of SHP-1/GAPDH at each time point was determined from phosphoimage analysis of the filters, relative to t=0 being set as 1.

SHP-1 enhances G-CSF-induced differentiation at the expense of proliferation in 32D cells
The kinetics of SHP-1 induction suggested a possible role for this protein in the termination of the proliferative phase of 32D[WT] cells stimulated with G-CSF. To directly examine whether SHP-1 could influence G-CSF-mediated growth and differentiation we introduced vectors expressing either wild-type SHP-1 (SHP-1^{wt}) or a catalytically-inactive mutant (SHP-1^{mut}) (Figure 2A) into 32D[WT] cells. Several clones were obtained which showed considerable over-expression of SHP-1^{wt}, as well as clones with significant expression of SHP-1^{mut} (Figure 2B). Northern blot analysis confirmed expression of both exogenous and endogenous SHP-1 in these 32D[WT/SHP-1^{wt}] and 32D[WT/SHP-1^{mut}] clones (data not shown).

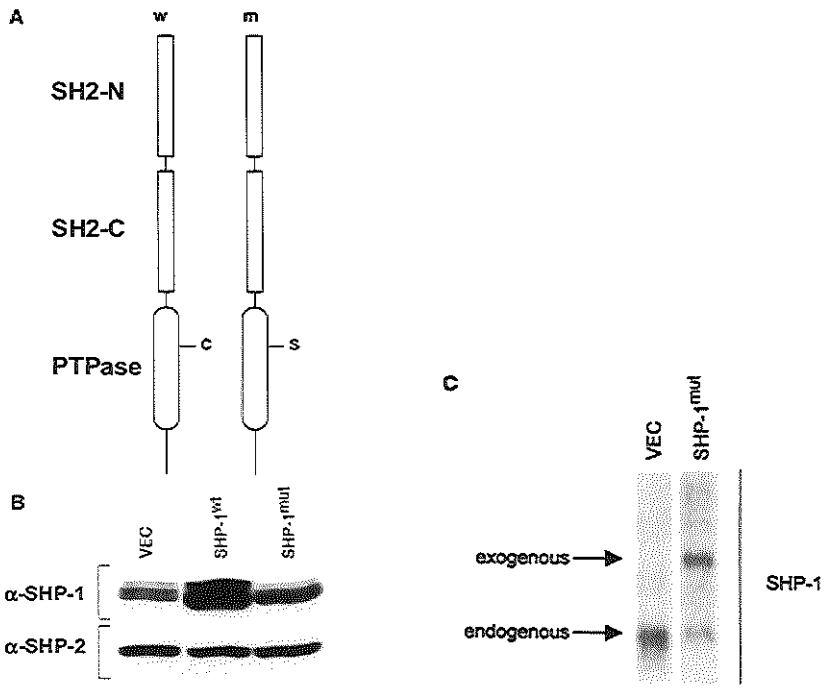

Figure 2. Expression of wild-type and mutant SHP-1 in 32D cells.
(A) Structure of wild-type (wt) and catalytically-inactive C453S mutant (mut) SHP-1.
(B) Western blot analysis of total lysates from representative 32D[WT] clones containing empty vector (VEC), or expressing SHP-1^{wt} or SHP-1^{mut}, probed with the antibodies indicated.

To assess the effects of SHP-1 expression, these clones were compared to vector control transfectants (32D[WT/VEC]) with respect to long-term growth and differentiation responses to G-CSF. 32D[WT/SHP-1^{wt}] clones showed reduced proliferation, while 32D[WT/SHP-1^{mut}] clones showed enhanced proliferation relative to 32D[WT/VEC] cells (Figure 3A). Interestingly, 32D[WT/SHP-1^{wt}] clones showed more complete maturation, while the 32D[WT/SHP-1^{mut}] clones were reduced in their maturation (Figure 3B and C).

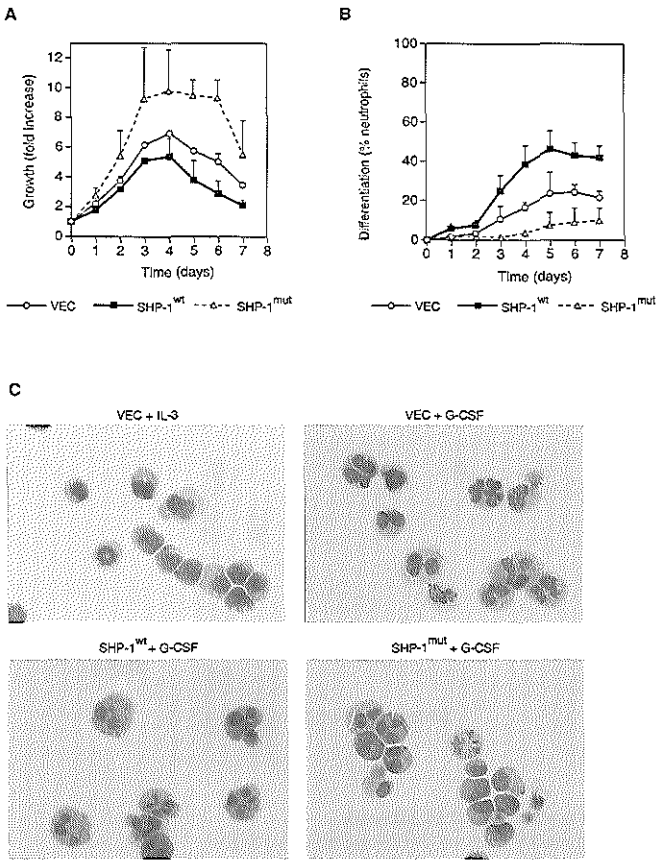

Figure 3. Effect of SHP-1 expression on growth and neutrophilic differentiation of 32D clones.
(A) G-CSF-mediated cell-proliferation of 32D[WT] clones containing empty vector (VEC) or expressing SHP-1^{wt} or SHP-1^{mut}, as indicated. Data represent mean growth of four clones, with standard error indicated.
(B) G-CSF-mediated maturation of 32D[WT] clones containing empty vector (VEC) or expressing SHP-1^{wt} or SHP-1^{mut}, described in Fig. 2C. Data are expressed as the percentage of living cells showing terminal neutrophilic differentiation at each time point.
(C) Morphological features of representative 32D[WT] clones containing empty vector (VEC) or expressing SHP-1^{wt} or SHP-1^{mut}, maintained in IL-3 or cultured for 6 days in the presence of G-CSF, as indicated.

STAT activation from the G-CSF-R is reduced by SHP-1

To investigate whether receptor signaling was directly affected by SHP-1, we analyzed STAT5 activation as a sensitive assay of receptor activation [16]. In addition, STAT5 appears to play a key role in G-CSF-induced mitogenesis [55,56]. 32D[WT/SHP-1^{wt}] clones showed reduced levels of STAT5 activation, while 32D[WT/SHP-1^{mut}] clones showed enhanced activation compared to 32D[WT/VEC] clones (Figure 4).

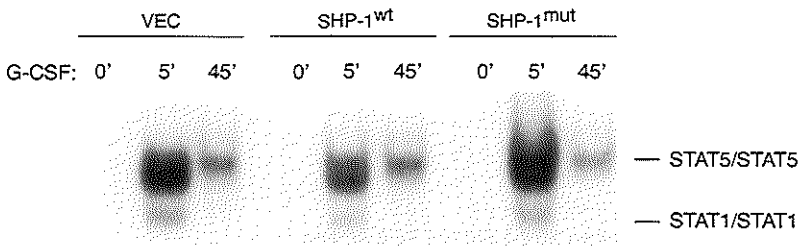

Figure 4. Effect of SHP-1 on G-CSF-mediated STAT activation.
EMSA of nuclear extracts from representative 32D[WT] clones containing empty vector (VEC) or expressing SHP-1^{wt} or SHP-1^{mut}, as indicated. Serum- and growth factor-deprived cells were incubated at 37°C without factor (-), with 100 ng/ml G-CSF (+) for the times indicated. Nuclear extracts were prepared and incubated with ^{32}P-labeled double-stranded β-cas oligonucleotide probe. The positions and identities of the different STAT complexes [16] are shown.

Tyrosine docking sites on the G-CSF-R are not required for the effects of SHP-1

Since SHP-1^{wt} affected G-CSF-R signaling, we analyzed whether it might directly associate with the activated G-CSF-R via its SH2 domains, as shown for a number of other receptors [37,39-41]. To this end, we produced the cytoplasmic domain of the human G-CSF-R in a recombinant, tyrosine-phosphorylated form. This was ^{32}P-labeled and used in an *in vitro* binding assay on various GST-SH2 domain fusions, which had been immobilized on nitrocellulose (Figure 5). The tyrosine-phosphorylated receptor did not bind to either SH2 domain of SHP-1, although it bound strongly to the N-terminal SH2 domain of SHP-2, suggesting there is no direct docking of SHP-1 to the activated G-CSF-R.

To confirm that receptor tyrosines were not required for the effects of SHP-1, we also introduced SHP-1^{wt} into Ba/F3 cells expressing either wild-type G-CSF-R (Ba/F3[WT] cells), or a mutant of the G-CSF-R that lacks all of the cytoplasmic tyrosines of the receptor (Ba/F3[mO] cells) (Figure 6A). Both of these lines are able to proliferate in response to G-CSF, although the Ba/F3[mO] cells do so at a slightly reduced rate [15]. Ba/F3[WT] clones over-expressing SHP-1^{wt} and SHP-1^{mut} were confirmed by Western blot analysis compared to cells into which just empty vector (VEC) was introduced (Figure 6B). When the growth of the various clones was compared, it was apparent that Ba/F3[WT/SHP-1^{wt}] clones showed reduced G-CSF-mediated proliferation compared to Ba/F3[WT/VEC] clones, while Ba/F3[WT/SHP-1^{mut}] clones showed an enhanced growth-rate (Figure 7). Clones over-expressing SHP-1^{wt} were also obtained in Ba/F3[mO] cells (Figure 6B). These also showed inhibition of G-CSF-dependent growth (Figure 7), indicating that the receptor tyrosines are not a prerequisite for the growth modulatory effects of SHP-1 on G-CSF signaling.

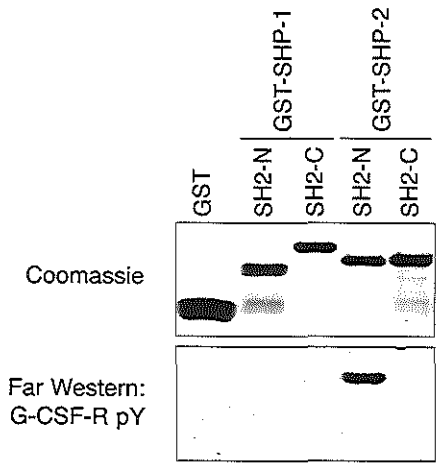

Figure 5. Lack of interaction between SHP-1 and G-CSF-R *in vitro*.
GST or GST-fusions of the SH2 domains indicated were separated on duplicate SDS-PAGE gels and either stained with Coomassie or transferred to nitrocellulose and subjected to Far-Western blot analysis using ^{32}P-labeled, tyrosine-phosphorylated G-CSF-R cytoplasmic domain (G-CSF-R pY) as a probe.

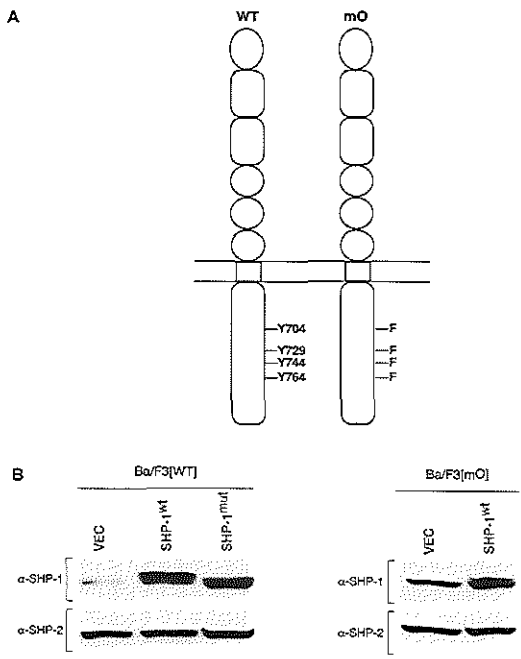

Figure 6. Expression of SHP-1 in Ba/F3 clones.
(A) Structure of wild-type (WT) and tyrosine null mutant (mO) G-CSF-Rs.
(B) Western blot analysis of total lysates from representative Ba/F3[WT] and Ba/F3[mO] clones containing empty vector (VEC) or vectors expressing SHP-1^{wt} or SHP-1^{mut}, probed with the antibodies indicated.

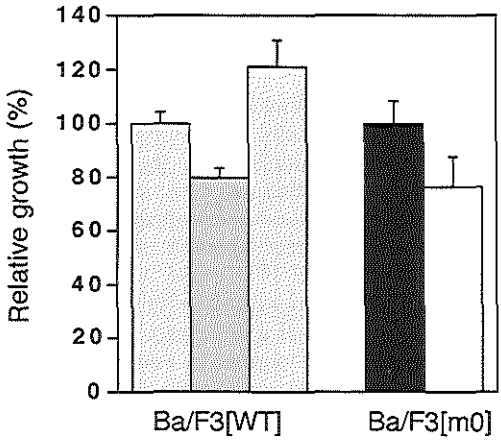

Figure 7. Effect of SHP-1 expression on growth of Ba/F3 clones.
Growth of indicated Ba/F3 clones on 100 ng/ml G-CSF, expressed relative to growth on IL-3 being set at 100 %. Data represent the mean and standard error for 3 clones of each.

Discussion

We set out to identify key regulators of the switch from proliferation to differentiation in G-CSF-stimulated myeloid 32D cells. We showed firstly that SHP-1 is up-regulated at the protein level following G-CSF stimulation of these cells. The kinetics of this induction largely mirrored the slowdown of the proliferative phase, suggesting a possible role for SHP-1 in the shut-off of proliferation during the terminal differentiation of these cells. Introduction of SHP-1^{wt} and SHP-1^{mut} expression vectors into 32D and Ba/F3 cells provided clear evidence that SHP-1 acts as a direct negative regulator of G-CSF-mediated proliferation. Unexpectedly, it appears that SHP-1 also augments differentiation in our 32D cell model, suggesting that it may not merely function as a negative regulator of growth-factor-induced mitogenesis, as other studies have suggested. Finally, we showed that receptor tyrosines were not required for SHP-1 to exert its modulatory effect on G-CSF-R signaling. This was supported by the lack of *in vitro* binding between the SH2 domains of SHP-1 and a recombinant, tyrosine-phosphorylated G-CSF-R cytoplasmic domain.

Motheaten and *viable motheaten* mice, which possess mutations in the gene for SHP-1, show an increased number, size and sensitivity of CFU-GM colonies, and an increased proliferation of bone marrow cells in response to G-CSF [30,33]. In addition, there is an accumulation of morphologically and phenotypically immature granulocytes [30,34,35], implying a role for SHP-1 in granulocytic differentiation. However, it was unclear from these studies whether the abnormalities in the granulocytic compartment of *motheaten* mice were a consequence of aberrant G-CSF signaling, which would directly impact on granulocytic differentiation, or of dysregulation of the signaling of other cytokines, such as IL-3 and GM-CSF, which promote myeloid proliferation and might therefore indirectly affect granulocytic differentiation. Our data showing that granulocytic differentiation is associated with increased SHP-1 expression and, importantly, that increasing SHP-1 levels through ectopic expression augments G-CSF-induced granulocytic differentiation, provides the first evidence for a functional role of SHP-1

in directly regulating this process by modulating G-CSF-R signaling. Thus, the immaturity of the granulocytic cells in the SHP-1-deficient mice is likely due, at least in part, to a defect in the (G-CSF-R-mediated) differentiation-promoting activity of the phosphatase.

Tapley *et al.* have previously shown both enhanced proliferative responses and increased STAT activation from bone marrow cells of *viable motheaten* mice stimulated with G-CSF [33]. These authors hypothesized that these results were simply due to the presence of more G-CSF-responsive precursor cells in the bone marrow of these animals, rather than a direct effect on G-CSF-R signaling. However, our data using clones overexpressing SHP-1^{wt} and SHP-1^{mut} provide clear evidence that SHP-1 acts as a direct regulator of G-CSF-mediated responses. Furthermore, the phenotype of the 32D[SHP-1^{mut}] clone, showing enhanced proliferation and STAT activation in response to G-CSF, resembles that of bone marrow cells from *viable motheaten* mice [33]. Therefore, it appears that a deficiency in SHP-1 activity alone is sufficient to explain an enhancement of G-CSF signals in responsive cells; any increase in the number of G-CSF-responsive cells in *viable motheaten* mice would further accentuate this effect.

The G-CSF-mediated increase in SHP-1 protein levels seen in 32D cells was not reflected in a concomitant increase in the levels of SHP-1 mRNA, suggesting some form of post-transcriptional control is removed by G-CSF-stimulation. Since SHP-1 levels in SHP-1^{wt} clones were only marginally increased by G-CSF treatment (data not shown), presumably reflecting an increase in the endogenous protein, it seems likely that the control is at the level of translation, with the sequences responsible being removed in the expression construct. We also examined SHP-1 levels in NB4 cells which were induced to differentiate into granulocytes by treatment with ATRA. In this case, levels of SHP-1 protein *and* mRNA were increased approximately 2-fold within one day (data not shown). This not only suggests that alternate means to control SHP-1 expression are possible, but also supports our hypothesis that increasing SHP-1 expression levels is important in regulating granulocytic differentiation. Interestingly, it has been reported that differentiation of HL-60 cells into macrophages [57] or granulocytes [58] is also associated with increased SHP-1 expression, while lower levels of SHP-1 expression have been found in certain normal immature B cells [59]. Modulation of SHP-1 expression might, therefore, play a more general role in the differentiation of multiple hematopoietic lineages.

We also showed that the effects of SHP-1 on G-CSF-R signaling did not require receptor tyrosines, and that SHP-1 does not bind directly to the activated G-CSF-R. This is in agreement with the data of Tapley *et al.*, showing that SHP-1 fails to associate with the G-CSF-R in co-immunoprecipitations [33]. Thus, SHP-1 appears able to modulate G-CSF-R signaling in the absence of direct docking to the receptor itself. Presumably this is through recruitment to other components of the receptor complex, although extensive co-immunoprecipitation studies have failed to identify these protein(s) (data not shown).

Finally, while our data shows that SHP-1 augments differentiation - leading to both earlier and more complete differentiation – it remains unclear exactly how this is achieved. SHP-1 could have a positive regulatory role in control of differentiation signals themselves. Alternatively, SHP-1 may be important for cell survival, which could also contribute to the observed effects on differentiation. In support of this possibility, it was extremely difficult to isolate clones over-expressing the catalytically-inactive mutant of SHP-1, and those which were obtained showed a clear tendency for reduced survival, especially at high cell densities

(data not shown). However, irrespective of the exact mechanistic details, this represents a new role for SHP-1, previously regarded primarily as a negative regulator of mitogenic signaling.

Conclusion

We report that the SH2 domain-containing protein tyrosine phosphatase SHP-1 is upregulated at the protein level during the granulocyte colony-stimulating factor (G-CSF)-mediated granulocytic differentiation of myeloid 32D cells. Enforced expression of SHP-1 in these cells decreases proliferation and enhances differentiation, while transfection of a catalytically-inactive mutant yields the opposite effect. Expression studies in cell lines harboring different G-CSF-R forms and *in vitro* binding analysis suggest that SHP-1 directly modulates G-CSF-mediated responses in hematopoietic cells via a mechanism that does not require direct docking to the activated G-CSF-R.

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Chapter 5

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

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FEBS Letters (2001) **503** 163-167

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.

Introduction

The somatostatin 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 somatostatin 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.

Materials and methods

Plasmid construction

Human SSTR2 cDNA (a kind gift from Dr G.I. Bell, Howard Hughes Medical Institute, Chicago IL) 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'- AGGACCGCGGCTAATGG-AGACCTCCAAAC), SH2/1172LL-R (5'-TCCATTAGCCGCGGTCCTCTGGGTCTCCG), T3-R (5'-GCGCAATTAACCCTCACTAAAGGG), Δ342-F (5'-GGAGTGACTGAAA-GCAGGACAAATCC) 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 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 *Bste*II and *Sal*I and cloned into pBABE containing wt-SSTR2 which had also been digested with these enzymes. The authenticity of all mutants was verified by restriction enzyme analysis and DNA sequencing.

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-□342], using a Progenitor apparatus set at 230 V, 100 μF, and 1 sec. After 48 hours of incubation, cells were selected with puromycin at a concentration of 1 μg/ml. Multiple clones were expanded for further analysis.

Flow cytometry

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

Internalization experiments

The SST analog [Tyr^3]octreotide (Novartis Pharma, Basel, Switzerland) was ionidated with ^{125}I by chloramine-T method and purified by HPLC, as described previously in detail [18]. The specific radioactivity of the radioligand was approximately 2000 Ci/mmol. Internalization of 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% 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 incubation for 10 min at RT. 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) as described above.

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).

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). Cells (1-2 x 10^5) 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 hours. Cells that had migrated into the lower chamber were counted using a cell counter (CASY®1/TTC, Schärfe Systems, Germany).

Results and discussion

Expression of SSTR2 and SSTR2 mutants on myeloid 32D cells

The structural features of two mutants of SSTR2, SSTR2-Δ342 and SSTR2-L360/361A are shown in Fig. 1A. Upon transfection in 32D cells, membrane expression of SSTR2 wild type 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 analysis (Fig. 1B).

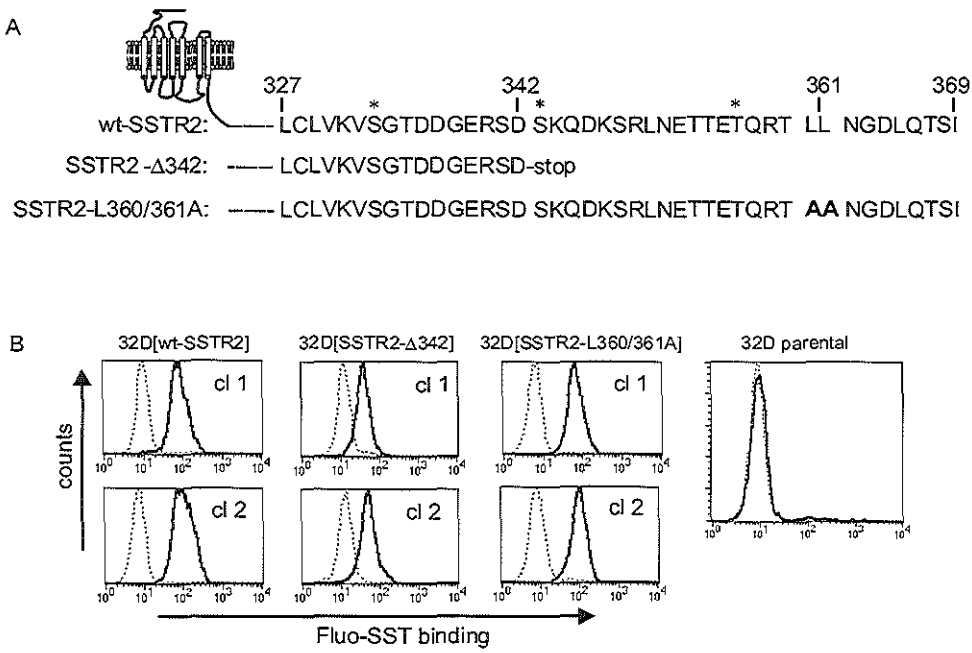

Figure 1. Expression of SSTR2 constructs in 32D cells. **(a)** The carboxyl-terminal sequences of SSTR2 and position of mutations. Asterisks indicate potential PKA/PKC phosphorylation sites. **(b)** Flow cytometric analysis of receptor expression on representative clones of 32D[wt-SSTR2], 32D[ΔSSTR2], 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 nonlabeled SST.

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.

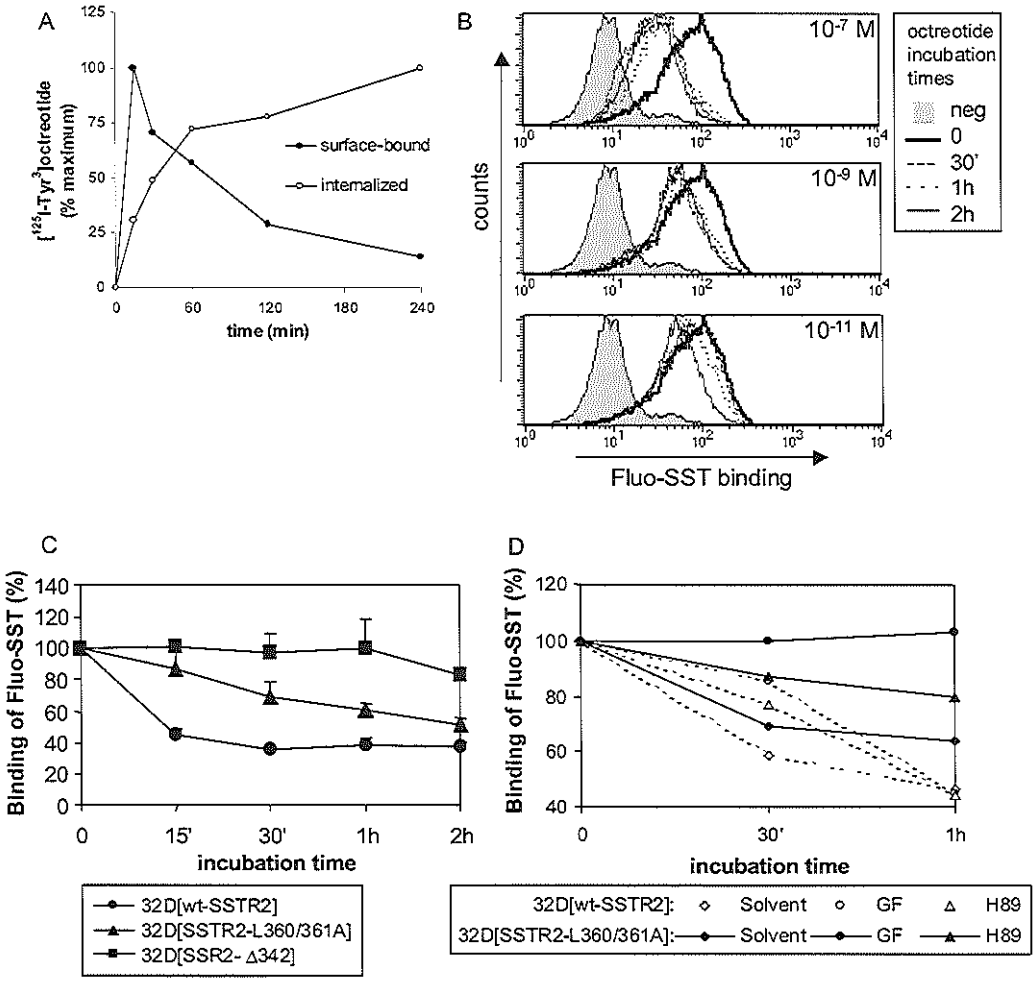

Figure 2 Internalization of SSTR2 mutants. **(a)** Time course of membrane binding and internalization of [^{125}I-Tyr^3]octreotide by 32D[wt-SSTR2] cells. Values are expressed as percent of maximum specific binding and internalization (maximum surface-bound: 2.3%, maximum internalization: 78%). Representative graph is shown from three independent experiments. **(b)** FACS analysis of octreotide-induced internalization of SSTR2. 32D[wt-SSTR2] cells were incubated with octreotide for 30', 1h and 2h 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 nonlabeled SST to determine nonspecific 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' and 1h at 37°C and further treated as under (c). A representative graph of 3 independent experiments is shown.

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 expression 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 GPCRs [8]. These kinases include the second-messenger kinases PKA and PKC. However, both the protein kinase A and protein kinase C inhibitors H89 and GF109203X only partly reduced octreotide-induced internalization of wt-SSTR2 (Fig. 2D).

The C-terminus of SSTR2 also comprises a di-leucine 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 di-leucine motif and PKA or PKC-mediated phosphorylation cooperate in the internalization of SSTR2 (Fig. 2D). Di-leucine 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.

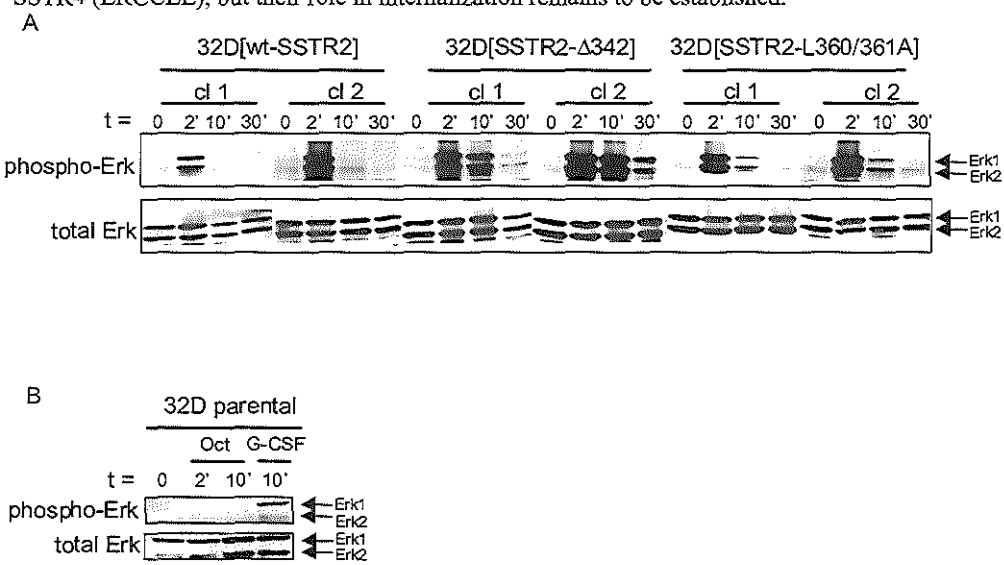

Figure 3. Octreotide-induced phosphorylation of Erk 1 and Erk2 in **(a)** 32D[wt-SSTR2], 32D[SSTR2-Δ342], 32D[SSTR2-L360/361A] **(b)** and 32D parental cells. Cells were treated with 10^{-6} M octreotide at 37°C for the times indicated. As a positive control, stimulation with 100 ng/ml granulocyte-colony 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.

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 nonselective 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).

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

Somatostatin 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-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 proteosomal 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.

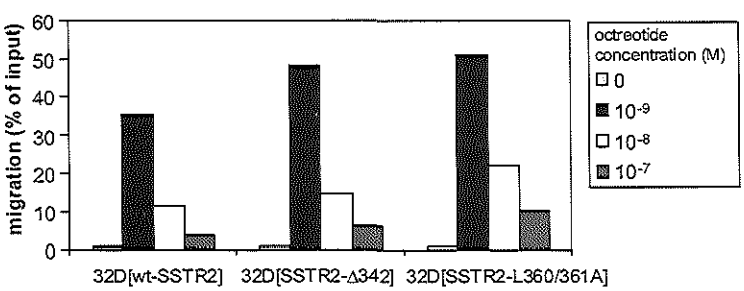

Figure 4. Migration of 32D[wt-SSTR2], 32D[SSTR2-Δ342] and 32D[SSTR2-L360/361A] cells in response to increasing concentrations of octreotide. A representative graph of three experiments is shown.

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Chapter 6

GENERAL DISCUSSION

6.1 Role of SST in hematopoiesis

The neuropeptide somatostatin (SST) participates in a variety of biological processes including neurotransmission, and inhibition of hormone secretion and cell proliferation. SST acts via a specific group of G protein-coupled receptors of which five subtypes have been identified (SSTR1-5) [1-3]. Previously, SSTRs have been detected in human lymphoid tissues, lymphoid cell lines and peripheral blood cells [4-11]. SSTRs were also found on cells derived from several hematological malignancies, including malignant lymphomas [4,12], acute myeloid leukemias (AML) and lymphoblastic leukemias [13,14]. While these findings suggested that both normal and malignant blood cells are responsive to SST, little was known about which blood cell functions are specifically modulated by the peptide. In fact, only moderate growth inhibitory effects of SST on some of these cell types were seen. Importantly, these studies did not address whether SST might be involved in the control of blood cell development by acting on the more primitive subset(s) of hematopoietic cells. The studies presented in this thesis were performed to address the following questions:

1. Do hematopoietic progenitor cell subsets and stem cells, as defined by immunophenotyping and *in vitro* culture technology, express SSTR and if so, which receptor subtype?
2. What is the functional significance of SSTR in early stages of blood cell development?
3. To what extent are SSTR expression and SST-induced responses in leukemic myeloid progenitor cells from AML patients different from normal?

1. Expression of SSTR on hematopoietic stem cell and progenitor cell subsets

In Chapter 2 we showed that, of the 5 SSTR subtypes, SSTR2 is exclusively expressed in human bone marrow cells. Interestingly, SSTR2 is present on a small subset (<1%) of cells. Immunophenotypic analysis showed that this subpopulation represents the $CD34^+$ fraction, with the $CD117^+$ (c-kit^+) subset of $CD34^+$ cells showing the highest expression. Whether this fraction comprises the most primitive stages of hematopoietic differentiation, including the pluripotent stem cells and early lineage committed progenitor cells, remains to be investigated. In order to accomplish this, we can determine CAFC (cobble stone area forming cell) and LTC-CFC (long-term culture colony-forming cell) subsets in bone marrow cells after sorting $SSTR2^+$ cells. *In vivo* transplantation of $SSTR2^+$ bone marrow cells in lethally irradiated mice (*in vivo* long-term repopulation assay) might assess the long-term repopulating ability *in vivo*.

2. Functional significance of SSTR in early stages of blood cell development

a.) Growth inhibition

We studied the mechanisms underlying SST responses in a myeloid cell line (mouse 32D cells) stably expressing SSTR2 and G-CSF receptors (Chapter 4.1). In this model, SST and octreotide reduced G-CSF-induced proliferation by approximately 50%. Incubation with octreotide significantly increased the activity of SHP-1 in these cells. Data presented in Chapter 4.2 show that the SHP-1 protein level is up-regulated during the G-CSF-mediated granulocytic differentiation of myeloid 32D cells. The combined data from Chapters 4.1 and 4.2 have been fitted into a model in which SSTR2 recruits SHP-1 to the plasma membrane, where it can down-modulate proliferative signals from the G-CSF receptor. Because SHP-1 does not directly bind to the G-CSF receptor (Chapter 4.2) we postulate that SHP-1 associates directly with Jak2 leading to dephosporylation of Jak2. However, since we have been unable to show an association between SHP-1 and Jak2, an alternative possibility is that SHP-1 affects other kinases, for example those of the Src family.

The antiproliferative effects of octreotide and SST have thus far only been demonstrated in malignant hematopoietic cell types and in certain hematopoietic cell lines. It remains to be established whether SST affects the proliferation of normal hematopoietic progenitors. Long term administration of radiolabeled octreotide for the treatment of neuroendocrine tumors showed a transient decline in platelet counts and lymphocyte subsets of some patients [15,16]. While this could suggest that octreotide has an inhibitory effect on the precursors of platelets and lymphocytes, it appears more likely that the drop in blood levels resulted from the long term radiation, rather than from direct antiproliferative effects of octreotide. In fact, we have thus far not obtained indications for antiproliferative effects of SST or octreotide on normal hematopoietic precursors.

b.) Migration

The experiments presented in Chapter 2 show that octreotide acts as a potent pro-migratory stimulus for $CD34^+$ BM cells *in vitro*. Using the 32D cell line model we could show that octreotide acts predominantly as a chemoattractant, but also has some chemokinetic activity. A major question that still needs to be addressed is how SST affects hematopoietic stem cell and progenitor cell migration *in vivo*, for instance following stem cell transplantation. In addition, it will be of interest to investigate whether SST may play a role in the migration of hematopoietic stem cells, which takes place during several distinct stages of fetal development. This phenomenon of targeted migration via the circulation to a specific tissue is referred to as "homing" [17]. While migration of stem cells from the fetal liver to the bone marrow crucially depends on SDF-1/CXCR4 [18], it is conceivable that other homing molecules are also involved. In addition, such alternative homing molecules might be more preferentially involved in other trajects of stem cell migration such as from the AGM region to the fetal liver. Whether SST/SSTR2 plays a significant role in the routing of stem cells during distinct stages of fetal development remains an important goal for further study.

Our *in vitro* data have established that SST exerts unique migration-inducing effects on normal and leukemic hematopoietic progenitors. These data raise the possibility that SST-induced migration may play a role in the homing and trafficking of these cells to different organs, and specific niches therein, during normal development and in pathological conditions. Unfortunately, due to different genetic background between SSTR2 +/+ and SSTR2 -/- mice we were unable to take this hypothesis a step further by investigating the role of SSTR2 in the homing of hematopoietic progenitors to the bone marrow *in vivo*. In a separate approach, we investigated whether injection of high dosages of SST could induce mobilization of hematopoietic progenitor cells from the bone marrow to the peripheral blood. However, instead of elevated levels of progenitor cells in the blood, the amount of progenitor cells dropped dramatically after injecting the mice with octreotide. This effect is likely to be caused by the significant vasoconstrictory effects of somatostatin on the blood vessels [19].

Whereas SDF-1- or CXCR4-deficient mice die perinatally with profound defects in the hematopoietic system, including severely reduced B-lymphopoiesis and defective myelopoiesis in both fetal liver and in bone marrow [18], SSTR2-deficient mice do not suffer from major hematopoietic defects. This could be a reflection of the restricted expression of SSTR2 to a small subpopulation in the BM ($CD34^+$/c-kit^+). SSTR2 may have a specific function that only becomes apparent when mice are submitted to some form of hematopoietic stress or under pathological conditions. The concentration of circulating SST might increase, and under such circumstances the migration of hematopoietic progenitors could get disturbed. Alternatively, SSTR2 may function as co-factor for CXCR4, with CXCR4 being the most prominent chemoattractant receptor.

A hypothetical scheme summarizing the actions of SST in normal hematopoiesis is shown in Figure 1.

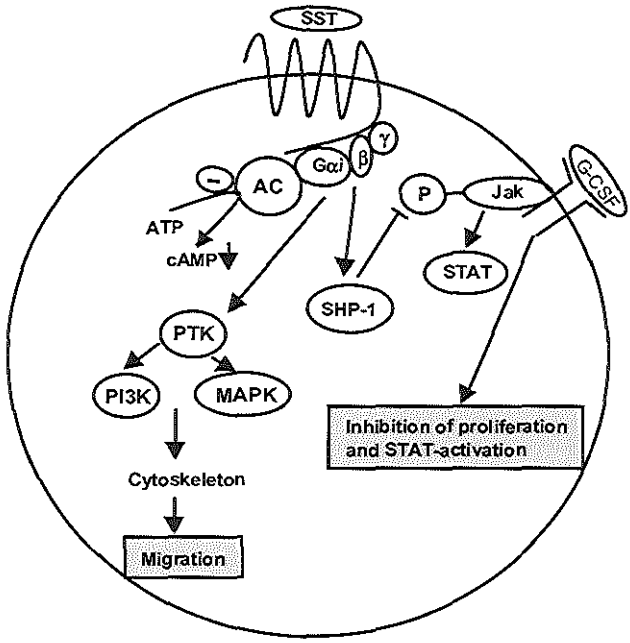

Figure 1. Hypothetical scheme for the actions of SST in normal hematopoiesis. Inhibition of G-CSF-induced proliferation via recruitment of SHP-1 to the cell membrane by SSTR2. SST-induced migration depending on cAMP levels, activation of MAPK and PI3K. SST: somatostatin; G-CSF: granulocyte colony-stimulating factor; Jak: Janus kinase; STAT: signal transducer and activator of transcription; SHP-1: Src homology 2 domain-containing protein-tyrosine phosphatase; MAPK: mitogen-activated protein kinase; PTK: protein tyrosine kinase; PI3K: phosphatidylinositol 3-kinase; AC: adenylate cyclase; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate.

3. To what extent are SSTR expression and SST-induced responses in leukemic myeloid progenitor cells from AML patients different from normal?

Like in normal bone marrow, SSTR2 is also expressed on AML cells (Chapter 3). Importantly, in contrast to normal human bone marrow, the SSTR2 expression is not restricted to $CD34^+/CD117^+$ in AML cells, which might suggest that SSTR2 expression in AML is not as tightly regulated as in normal cells. More than 50% of all AML samples we investigated showed SSTR2 expression. Despite the aberrant expression of SSTR2 on AML cells we observed that SST also induced migration of AML cells in the same concentration-dependent manner. These data suggest that somatostatin may have an effect on the homing and migration of normal as well as AML cells *in vivo*, with possible implications for the clinical application of SST and its analogs. Our finding that somatostatin and octreotide influence the migratory behavior of AML blasts suggests that treatment with these peptides may also have consequences for compartmentalization and spreading of the leukemic cells *in vivo*.

6.2 Comparison of SST with other chemoattractants

The first member of the chemokine family to demonstrate the capacity for direct movement (chemotaxis) of $CD34^+$ cells and hematopoietic progenitor cells (HPC) was stromal cell-derived factor-1 (SDF-1) [20]. Presently, only few other chemokines, of a large number assayed, have been demonstrated to cause chemotaxis of progenitor cells. These chemokines, macrophage-inflammatory protein (MIP)-3α/EBI1-ligand chemokine/CKβ-11 (MIP-3α) [21] and exodus-2 [22], have a more restricted target cell population confined to CFU-M. This contrasts with SDF-1, a broad range chemoattractant for most types of HPC tested.

One of the best studied molecules among the chemokines is interleukin-8 (IL-8), which is involved in chemotaxis and activation of neutrophils [23]. *In vivo* administration of IL-8 induces granulocytosis and the release of immature white blood cells into the circulation (mobilization). Two types of IL-8 receptors have been reported, CXCR1 and CXCR2 [24]. Unlike SSTR2, IL-8 receptor is only expressed on mature leukocytes (granulocytes, monocytes and mast cells).

Since CXCR4 and SSTR2 receptors are present on the same hematopoietic progenitor ($CD34^+$) cells, their responses are likely to be cross-regulated. In Chapter 2 we have shown that SDF-1 indeed antagonized octreotide-mediated chemoattraction and *vice versa*. There are two possible explanations for this finding. One explanation is that receptor cross-desensitization is involved, as has recently been demonstrated for a variety of chemoattractant receptors [25]. Another explanation is that the chemotaxis is affected by a change in cell polarization caused by preincubation with heterologous ligand. Irrespective of the underlying mechanism(s), the observation that SDF-1 and SST act as each other's antagonist may have significant implications for the migratory behavior of hematopoietic progenitor cells under certain physiological and pathological conditions. A competition between the two different chemoattractants may become apparent. Depending on the gradient or concentration of SST or SDF-1 on the one hand, and the distribution of the receptors on the cell surface on the other hand, a cell may decide the direction of migration.

6.3 Regulation of somatostatin receptor subtype 2 in hematopoietic cells

After activation, G protein-coupled receptors become either partially or totally desensitized to repeated stimulation with the same agonist. This process is thought to involve phosphorylation of serine and threonine residues in the C-tail of the receptor and receptor sequestration by internalization. Desensitization may be critical for maintaining the capacity of the cell to sense a chemoattractant gradient. In the case of SST, the processes of desensitization and internalization are of considerable clinical importance, as stable peptide analogs of SST are used for the long term treatment of hypersecretory neuroendocrine tumors of the pituitary gland and the pancreas [26]. These analogs not only inhibit hormone secretion, but also have an antiproliferative effect on tumor cells [27]. Internalization of SST is of special importance when radiotherapy of certain SST-positive human cancers with isotopes coupled to SST analogs is considered. The accurate evaluation of SSTR subtype status in hematological diseases, like AML, might become of value. In order to characterize the mechanism of SSTR2 internalization and to identify determinants in the SSTR2 C-tail that are responsible for mediating receptor internalization in hematopoietic cells, we generated transfectants of the myeloid 32D cell line stably expressing either wild-type SSTR2, a C-terminally truncated mutant or a dileucine mutant, and analyzed the ability of these mutant receptors to undergo agonist-induced internalization (Chapter 5). The results obtained from that study showed that multiple residues within the SSTR2 C-terminal tail appear to mediate octreotide-induced internalization in 32D cells.

Internalization of SST by tumors that overexpress SSTR opens the possibility of carrying out radiotherapy with somatostatin analogs coupled to radionuclides. Since the SST analogs mainly bind to SSTR2 and SSTR5 one can expect radiotoxic effects of radiotherapy on the hematopoietic system, leading to a decrease in early hematopoietic progenitors and a drop in blood parameters.

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SUMMARY

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SAMENVATTING (SUMMARY IN DUTCH)

Summary

Somatostatin (SST) is a neuropeptide with diverse functions in different organ systems, originally identified as a factor produced in the hypothalamus that inhibited the production of growth hormone from the pituitary. There are two biologically active forms: a 14-amino acid form (SST-14) and an N-terminally extended 28-amino acid form (SST-28). Somatostatin can act as a neurotransmitter or paracrine/autocrine regulator, or more widely via the circulation, to regulate such diverse physiological processes as cell secretion, neuromodulation, smooth muscle contractility, nutrient absorption, or cell growth. The diverse effects of SST are mediated via high affinity plasma membrane SST receptors (SSTR), which belong to a superfamily of seven-transmembrane-spanning G protein-coupled receptors. Five subtypes of human SST receptors (SSTR1-5) have been identified, which are encoded by related genes localized on different chromosomes. Recent studies have suggested that somatostatin inhibits proliferation of certain hematopoietic cell lines. However, little is known of the effects of somatostatin on primary hematopoietic stem cells and progenitor cells.

In **Chapter 1**, an overview is given of the expression and functional significance of somatostatin receptors, particularly in the hematopoietic system.

The presence of SSTR in normal mouse and human bone marrow is described in **Chapter 2**. Both in human and mouse, SSTR subtype 2 is expressed. In addition, in mouse bone marrow SSTR4 is also expressed. By flow-cytometry, using fluorescent somatostatin (Fluo-SST), we estimated that the expression of SSTR2 was restricted to an immature population ($CD34^+/c\text{-}kit^+$) in the bone marrow. To unravel the role of SSTR2 in hematopoiesis we performed *in vitro* migration assays. We established that SST and octreotide (a stable somatostatin analog) are chemoattractants for primitive hematopoietic progenitor cells and thus identified a novel positive regulatory role for this peptide. The mechanisms and pathways underlying migration mediated by SSTR2 were investigated in a myeloid cell model (mouse 32D cells) in which we overexpressed SSTR2.

We analyzed the expression and function of SSTR2 in acute myeloid leukemia (AML) cells of 16 patients (**Chapter 3**). SSTR2 mRNA was detected in all AML samples. In contrast to normal bone marrow, the expression of SSTR2 was not restricted to $CD34^+/c\text{-}kit^+$ cells. Like normal human bone marrow cells, AML cells migrate towards an octreotide gradient. The degree of migration correlated with the cell surface density of SSTR2 as measured by Fluo-SST binding. These findings indicate that SST-mediated mechanisms may influence the migratory behavior of AML blasts, which might have consequences for compartmentalization and spreading of the leukemic cells *in vivo*.

Earlier, inhibitory effects of SST and octreotide on the *in vitro* proliferation of AML cells have been reported. In **Chapter 4**, we studied the mechanisms underlying SST-induced responses in 32D cells stably expressing SSTR2 and G-CSF receptors. Based on two separate studies (**Chapter 4.1 and 4.2**) we present a model in which the protein tyrosine phosphatase SHP-1 plays a central role in the inhibitory effects of SST on G-CSF-induced proliferation. Stimulation of SSTR2 with octreotide leads to a reduction in G-CSF-induced STAT5 activation. In addition we observed stimulation of SHP-1 tyrosine phosphatase activity by octreotide. Together these data suggest that

the somatostatin receptor modulates G-CSF-mediated responses in myeloid cells through activation of SHP-1.

In **Chapter 5**, we sought to characterize the mechanism of SSTR2 internalization and identify determinants in the SSTR2 C-terminus that are responsible for mediating receptor internalization in hematopoietic cells. Therefore we generated transfectants of the myeloid 32D cell line stably expressing either wild-type SSTR2, a C-terminally truncated mutant or a dileucine mutant, and analyzed the ability of these mutant receptors to undergo agonist-induced internalization. The data presented in this study demonstrate that octreotide, a stable SST analog, rapidly induces internalization of SSTR2 when expressed in myeloid 32D cells. Truncation of part of the C-terminal tail of SSTR2 or mutation of a dileucine motif in the C-tail leads to a loss of or respectively delayed octreotide-induced internalization. In addition, we observed that octreotide-induced Erk phosphorylation is prolonged due to these mutations, indicating that inhibition of internalization leads to prolonged Erk activation. However, octreotide-induced migration is unaffected by these mutations. Finally, this study also provides evidence that internalization of SSTR2 is dependent upon PKC and PKA activities.

In **Chapter 6**, the findings described in this thesis are summarized and their significance for the understanding of the function of SSTR in hematopoiesis is discussed.

Samenvatting (Summary in Dutch)

Somatostatine (SST) is een neuropeptide met diverse functies in verschillende organen. SST is als eerste geïdentificeerd als een factor die geproduceerd werd door de hypothalamus en die de productie van groeihormoon door de hypofyse remde. SST heeft twee biologisch actieve vormen: een 14-aminozuur (SST-14) en een N-terminaal verlengde 28-aminozuur grote vorm (SST-28). SST kan werken als een neurotransmitter of paracriene/autocriene regulator. Maar SST heeft ook een uitgebreidere functie in de circulatie door de regulatie van diverse fysiologische processen, zoals cel secretie, neuromodulatie, contractie van gladde spiercellen, absorptie van voedingstoffen of cel groei. De diverse effecten van SST worden gemedieerd door hoge affiniteit plasma membraan receptoren (SSTR), die behoren tot een superfamilie van zeven-transmembraan G-eiwit-gekoppelde receptoren. Tot nu toe zijn vijf subtypen van humane SST receptoren (SSTR1-5) geïdentificeerd. Ze worden gecodeerd door verwante genen gelegen op verschillende chromosomen. Uit recente studies is gebleken dat somatostatine de proliferatie van bepaalde hematopoïetische cellijnen remt. Echter, er is weinig bekend van de effecten van somatostatine op primaire hematopoïetische stamcellen en voorloper cellen.

In **Hoofdstuk 1**, wordt een overzicht gegeven van de expressie en functies van somatostatine receptoren, in het bijzonder in het hematopoïetisch systeem.

De aanwezigheid van SSTR in normaal muis en humaan beenmerg wordt beschreven in **Hoofdstuk 2**. SSTR subtype 2 komt zowel in humaan als in muis beenmerg tot expressie. Bovendien, komt in muis beenmerg ook nog SSTR4 tot expressie. Met behulp van flow-cytometrie, gebruik makend van fluoriscent somatostatine (Fluo-SST), konden we aantonen dat de expressie van SSTR2 beperkt is tot een onrijpe populatie ($CD34^+$/c-kit^+) in het beenmerg. Met behulp van migratie assays hebben we de rol van SSTR2 in hematopoïese onderzocht. Daaruit volgde dat SST en octreotide (een stabiele analoog van somatostatine) chemoattractanten zijn voor primitieve hematopoïetische voorloper cellen. Hiermee hebben we een nieuwe positief regulerende rol voor dit peptide geïdentificeerd. Het mechanisme en de wegen die hieraan te grondslag liggen hebben we onderzocht in een myeloïd cellijn model (muizen 32D cellen) waarin we SSTR2 tot overexpressie gebracht hebben.

We hebben de expressie en functie van SSTR2 onderzocht in acute myeloïde leukemie (AML) cellen van 16 patiënten (**Hoofdstuk 3**). SSTR2 mRNA werd gevonden in alle AML samples. In tegenstelling tot normaal beenmerg, was de expressie van SSTR2 niet beperkt tot $CD34^+$/c-kit^+ cellen. Evenals in normaal beenmerg migreerde AML cellen richting een octreotide gradiënt. De mate van migratie correleerde met de hoeveelheid receptoren (SSTR2) op het celoppervlak. Deze bevindingen laten zien dat SST-gemedieerde mechanismen het migratie gedrag van AML blasten kunnen beïnvloeden, hetgeen gevolgen kan hebben voor de compartimentalisatie en spreiding van leukemie cellen *in vivo*.

Uit eerder onderzoek is het remmende effect van SST en octreotide op de *in vitro* proliferatie van AML cellen al gebleken. In **Hoofdstuk 4** hebben we de onderliggende mechanismen van de SST-geïnduceerde responsen onderzocht in 32D cellen waarin SSTR2 en G-CSF receptoren tot expressie zijn gebracht. We laten een model zien gebaseerd op twee verschillende studies (**Hoofdstuk 4.1 en 4.2**) waarin een proteïne

tyrosine fosfatase (SHP-1) een centrale rol speelt in het remmend effect van SST op G-CSF-geïnduceerde proliferatie. Stimulatie van SSTR2 met octreotide leidde tot een reductie van G-CSF-geïnduceerde STAT5 activatie. Tevens zagen we dat SHP-1 tyrosine fosfatase activiteit gestimuleerd werd door octreotide. Samen laten deze data zien dat de somatostatine receptor de G-CSF-gemedieerde responsen in myeloïde cellen moduleert door activatie van SHP-1.

In **Hoofdstuk 5** onderzochten we de mechanismen die betrokken zijn bij internalisatie van SSTR2 en bepaalden we de belangrijke gebieden in het C-terminale deel van SSTR2 die betrokken zijn bij receptor internalisatie in hematopoïetische cellen. Daarvoor genereerden we transfectanten van de myeloïde 32D cellijn die of wild-type SSTR2, een C-terminaal verkorte mutant of een dileucine mutant tot expressie brachten. In deze cellijnen onderzochten we of deze mutanten agonist-geïnduceerde internalisatie konden ondergaan. De gegevens van deze studie laten zien dat octreotide, een stabiele SST analoog, snelle internalisatie van SSTR2 induceert wanneer deze receptor tot expressie wordt gebracht in 32D cellen. Wanneer het C-terminale deel van SSTR2 wordt verkort of wanneer een dileucine motief in het C-terminale deel wordt gemuteerd leidt dit tot verlies van of vertraagde octreotide-geïnduceerde internalisatie. Tevens laten we zien dat octreotide-geïnduceerde Erk fosforylatie wordt verlengd door deze mutaties. Hetgeen betekent dat remming van internalisatie leidt tot verlengde Erk activatie. Echter, octreotide-geïnduceerde migratie is onveranderd door deze mutaties. Tenslotte, laat deze studie zien dat internalisatie van SSTR2 afhankelijk is van PKC en PKA activiteit.

In **Hoofdstuk 6** worden de bevindingen van dit proefschrift samengevat en de betekenis van SSTR in hematopoïese wordt bediscussieerd.

ABBREVIATIONS

AGM	aorta-gonad-mesonephros
AML	acute myeloid leukemia
BFU-E	burst forming unit-erythroid
BM	bone marrow
BMNC	bone marrow nucleated cells
bp	basepair
CAFC	cobble stone area forming cell
CD	cluster of differentiation
CytD	Cytochalasin D
CFC	colony forming cell
CFU	colony forming unit
CNS	central nerve system
CXCR	chemokine receptor
DbcAMP	dibutyryl cyclic adenosine monophosphate
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
ERK	extracellular-regulated kinase
FAB	French-American-British cytomorphological classification
FACS	fluorescence-activated cell sorting
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GRK	G protein receptor kinase
HCP	hematopoietic cell phosphatase
HGF	hematopoietic growth factor
IL	interleukin
Jak	Janus kinase
LTC	long-term culture
MAPK	mitogen-activated protein kinase
MF	mean fluorescence
Mut	mutant
NGF	nerve growth factor
NK-1	neurokinin 1
Oct	octreotide
PB	peripheral blood
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKB/akt	protein kinase B
PKC	protein kinase C
PRL	prolactin
PTP	phosphotyrosine phosphatase
PTX	pertussis toxin
R	receptor
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor (KL)
SDF-1	stromal cell-derived factor-1
SH	Src homology
SHP-1	Src homology 2 domain-containing protein-tyrosine phosphatase

SP (subP)	substance P
SST	somatostatin
SSTR	somatostatin receptor
STAT	signal transducer and activator of transcription
TSH	thyroid-stimulating hormone
TMD	transmembrane domain
VEC	vector control
wt	wild type

DANKWOORD

Graag wil ik iedereen bedanken die op wat voor manier dan ook heeft bijgedragen tot het ontstaan van dit proefschrift. Een aantal mensen wil ik toch bij naam noemen en bedanken.

Allereerst wil ik mijn twee promotoren, Ivo Touw en Bob Löwenberg bedanken. Ivo, je bent altijd enthousiast gebleven, ook op momenten waar we even niet wisten waar het onderzoek heen moest. Bedankt ook voor je kritische blik op alle versies van het manuscript (ik heb ze niet geteld). Bob, ook jij bent altijd een grote stimulans geweest gedurende het onderzoek. Tevens wil ik je bedanken voor de gesprekken die we gehad hebben over mijn toekomst. Daar heb ik heel veel aan gehad.

Professor Grootegoed, professor Dzierzak en professor Lamberts wil ik bedanken voor het kritisch doorlezen van mijn proefschrift. Professor Lamberts wil ik nog extra bedanken voor het schrijven van het NWO project en het enthousiasme wat hij altijd voor mijn onderzoek getoond heeft.

Dan natuurlijk de "G" groep, een hele leuke groep mag ik wel zeggen.
Alister, you have been a great colleague and friend. You always had the best ideas how to proceed with my research, which we mainly discussed during our fruitful coffeebreaks. Louise, je was er altijd wanneer ik een helpende hand nodig had. Van jou heb ik de fijne kneepjes van het celkweken geleerd. Nicole, bedankt voor je bijdrage aan het AML onderzoek. Claudia, zonder jou had ik dat muizen verhaal nooit voor elkaar gekregen. Gert-Jan en Judith, naast het feit dat jullie natuurlijk ontzettend aardig zijn, waren jullie ook nooit te beroerd om even te helpen. En verder natuurlijk Amrita, Yvette, Karin, John, Stephan, Bart, Mirjam en Antoinette (ondertussen alweer een Delwelletje).
En tenslotte mijn twee paranimfen, Daphne en Anita. Naast twee geweldige collega's zijn jullie ook nog eens de beste vriendinnen die iemand zich kan wensen.

De M&M groep. Martine en Marieke, bedankt voor al jullie hulp en ideeën.

Dan de Delwelletjes, ook een groep apart. Bedankt voor alle hulp en gezelligheid. Marieke, het was leuk samen hè in New Orleans? Nog een extra bedankje aan mijn kamergenoten, Derrick en Peter, de koffie was lekker.

Paula, hartelijk bedankt voor al je input. Zelden zo een georganiseerd iemand meegemaakt als jij.

Karola en Egied. Karola bedankt voor alle dia's die je voor me gemaakt hebt. Egied bedankt voor het mooie ontwerp van de cover, ook al was het op de valreep.

Interne Geneeskunde III. Leo, bedankt voor het meedenken en voor de organisatie rondom de muizen. Elgin, Peter, Marjolijn en Joke, bedankt voor alle hulp bij de experimenten. Martin, bedankt voor je enthousiasme.

En tenslotte natuurlijk pa en ma. Degenen die me altijd gestimuleerd hebben al vanaf de kleuterschool, toen ik nog moest leren mijn veters te strikken. Zonder hen was dit boekje er zeker niet gekomen. Bedankt voor alles.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 21 maart 1970 te Oosterhout geboren. Ze behaalde haar VWO diploma aan het Mgr. Frencken College te Oosterhout in 1988. Na het behalen van het HLO diploma te Etten-Leur in 1992, studeerde zij Biologie (Fysiologisch-biochemische richting) aan de Katholieke Universiteit Nijmegen. In september 1995 haalde zij haar doctoraal examen met als hoofdvak Celbiologie en als bijvak Tumorimmunologie. Tijdens de doctoraalstudie deed zij onderzoek naar de rol van een nieuw PDGF (platelet-derived growth factor) α-receptor transcript o.l.v. Prof. Dr. E.J.J. van Zoelen (Celbiologie, KUN, Nijmegen) en naar de regulatie van de adhesie receptor LFA-1 (leukocyte function-associated 1) o.l.v. Prof. Dr. C.G. Figdor en Dr. Y van Kooyk (Tumorimmunologie, academisch ziekenhuis St. Radboud, Nijmegen). In februari 1996 begon zij aan het in dit proefschrift beschreven onderzoek bij het Instituut Hematologie van de Erasmus Universiteit Rotterdam o.l.v. Prof. Dr. I.P. Touw en Prof. Dr. B. Löwenberg. Na haar promotie is zij werkzaam geweest als hoofd datamanagement bij de sectie Statistiek van de afdeling Trials en Statistiek van het Academisch Ziekenhuis Rotterdam.

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