THE ROLE OF GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTOR SIGNALING IN NEUTROPHIL DEVELOPMENT

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DE ROL VAN G-CSF RECEPTOR SIGNAAL TRANSDUCTIE IN DE ONTWIKKELING VAN NEUTROFIELE GRANULOCYTEN

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ter nagedachtenis aan *mijn moeder*
voor *mijn vader*

Contents

Abbreviations

AML	acute myeloblastic leukemia
Ara-C	cytosine arabinoside
C	carboxy
cdk	cyclin-dependent kinase
CRE	cAMP responsive element
CRH	cytokine receptor homology
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
EPO	erythropoietin
F	phenylalanine
GAP	GTPase activating protein
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione S-transferase
HA	hemagglutinin
HCP	hematopoietic cell phosphatase
HGF	hematopoietic growth factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Jak	Janus kinase
kD	kilodalton
K_d	dissociation constant
LIF	leukemia inhibiting factor
MAP	mitogen-activated protein
M-CSF	macrophage colony-stimulating factor
NF1	neurofibromatosis type 1
PDGF	platelet-derived growth factor
PTB	phosphotyrosine-binding
PTP	phosphotyrosine phosphatase
R	receptor
SCF	stem cell factor
SCN	severe congenital neutropenia
SH	Src homology
Sos	Son of sevenless
STAT	signal transducer and activator of transcription
TPO	thrombopoietin
WT	wild-type
Y	tyrosine

CHAPTER 1

Introduction

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1.1 Hematopoiesis

Hematopoiesis or blood cell formation is a strictly regulated process that, in adult individuals, takes place mainly in the bone marrow. 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 mature blood cells that include granulocytes, monocytes/macrophages, lymphocytes, erythrocytes, and platelets. Most mature blood cells have a limited life span and need to be replenished constantly. This continuous production is tightly balanced and is regulated essentially by two mechanisms. Stromal cells in the bone marrow affect hematopoiesis by direct cell-to-cell contact and provide a suitable microenvironment required for hematopoietic cell development. In addition, a network of cytokines and hematopoietic growth factors (HGF) specifically control the proliferation, differentiation, survival, and function of different hematopoietic cells. This network is particularly important under stress conditions, such as infection or bleeding, when a rapid rise in specific blood cell types is needed.

1.2 Hematopoietic growth factors

HGFs are glycoproteins that are produced by stromal cells, fibroblasts, endothelial cells, lymphocytes, monocytes and macrophages, and by specialized cells in various organs, such as kidney and liver. The levels of HGFs are normally low, but can be enhanced substantially in response to extracellular stimuli. HGFs can be roughly subdivided into early-acting lineage-nonspecific factors and late-acting lineage-specific factors. Some early-acting factors are interleukin (IL)-1, IL-3, IL-4, IL-6, IL-11, stem cell factor (SCF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). For instance, IL-3 stimulates the growth of multipotential progenitor cells that can develop into granulocytes, macrophages, erythrocytes, and megakaryocytes. However, IL-3 does not support the terminal stages of hematopoiesis, as IL-3 responsiveness of progenitor cells declines with differentiation (1). In contrast, late-acting HGFs, including IL-5, erythropoietin (EPO), thrombopoietin (TPO), macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF), are mostly lineage specific. They not only stimulate proliferation, but also induce terminal differentiation of hematopoietic cells of particular lineages. Further characteristic features of HGFs are their functional pleiotropy and redundancy. Many HGFs, in particular those acting at early stages of hematopoiesis, display overlapping biological activities on a variety of cell types. Conversely, different HGFs can act on the same cell type to mediate similar effects. The functional interplay between HGFs, either synergistically or antagonistically, will determine the cellular response.

1.3 G-CSF and granulopoiesis

Neutrophilic granulocytes are an essential component of the host defense system against infections. Mature neutrophils arise from bone marrow stem cells following a process involving proliferation, commitment to the granulocytic lineage, and terminal differentiation. Several HGFs, including IL-3, IL-6, SCF, GM-CSF, and G-CSF, have been shown to be positive regulators of granulopoiesis (2,3). Most of these HGFs support the proliferation of early myeloid progenitor cells and have only a limited ability to induce differentiation. G-CSF, however, not only stimulates the proliferation but also strongly induces terminal differentiation of granulocytic progenitor cells. G-CSF also activates certain functions of mature neutrophils and promotes their survival (4). G-CSF enhances the production of superoxide, alkaline phosphatase, and myeloperoxidase, the chemotactic activity and the antibody-dependent cellular cytotoxicity of neutrophils (5-9). Using serum-free culture systems, an absolute requirement for G-CSF for neutrophil colony formation has been shown (10). Neither IL-3 nor GM-CSF alone is capable of effectively supporting neutrophil colony formation. However, G-CSF can synergize with both IL-3 and GM-CSF to support neutrophil progenitor proliferation in culture. Thus, IL-3 and GM-CSF induce proliferation of progenitor cells in the early stages of development, whereas G-CSF is a neutrophil lineage-specific regulator that stimulates the terminal stages of neutrophil development.

In vivo studies in mice have indicated that G-CSF induces a much higher peripheral blood granulocyte count than does IL-3 or GM-CSF (3). The granulopoietic effects of G-CSF have been reproduced in humans and have led to its widespread clinical application in the setting of chemotherapy-induced neutropenia and bone marrow transplantation (11). Several observations have indicated that G-CSF is indispensable for normal granulopoiesis *in vivo*. In humans, low neutrophil counts coexist with high serum G-CSF levels, suggesting a regulatory role for G-CSF in maintaining steady state neutrophil production (12). Concurrent elevation in neutrophil numbers and serum G-CSF levels observed during infections implies a role for G-CSF as an emergency factor for granulopoiesis (13). Furthermore, dogs injected with human G-CSF that produced neutralizing antibodies cross-reactive against endogenous canine G-CSF developed prolonged neutropenia (14). Infusion of plasma from a neutropenic dog with anti-G-CSF antibodies into a normal dog also caused neutropenia. More recently, it was shown that mice lacking G-CSF due to targeted disruption of the *G-CSF* gene developed chronic neutropenia (15). Additionally, the granulopoietic response to infection was severely impaired in G-CSF-deficient mice. Collectively, these results indicate that G-CSF plays a key role in regulating granulopoiesis in both steady state and stress conditions.

1.4 G-CSF receptor: structure and subdomains

The biological effects of G-CSF are mediated through a specific receptor on the surface of responsive cells. The G-CSF receptor (G-CSF-R) is a member of the hematopoietin receptor superfamily to which the receptors for IL-2 to IL-7, IL-9, IL-11, IL-12, EPO, TPO, GM-CSF, leukemia inhibiting factor (LIF), growth hormone, prolactin, ciliary neurotrophic factor, and the gp130 signal transducing protein also belong (16). Characteristic structural features of members of this family are the presence of four highly conserved cysteine residues and a motif of tryptophan-serine-X-tryptophan-serine (WSXWS), within an approximately 200 amino acid region in the extracellular domain. This region is referred to as the cytokine receptor homology (CRH) region and is crucial for ligand binding. Molecular cloning showed that the murine and human G-CSF-R are single transmembrane proteins of 812 and 813 amino acid residues, respectively, with 62.5% homology at the amino acid level (17-19). The extracellular domain of the human G-CSF-R comprises 603 amino acids and is composed of an immunoglobulin-like sequence, a CRH region, and three

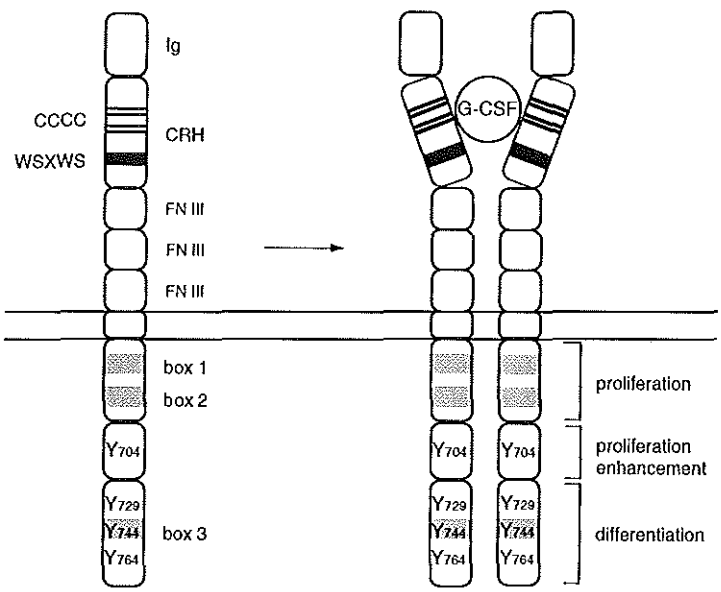

Figure 1. Schematic diagram of the human G-CSF-R. The extracellular domain is composed of an immunoglobulin (Ig)-like sequence, a cytokine receptor homology (CRH) region with four conserved cysteine (C) residues and a motif of tryptophan-serine-X-tryptophan-serine (WSXWS), and three repeats of the fibronectin type III (FN III) module. Receptor dimerization upon ligand binding initiates activation of signal transduction pathways via distinct cytoplasmic subdomains of G-CSF-R that will elicit the indicated diverse biological effects of G-CSF. Boxes 1, 2, and 3 denote cytoplasmic motifs conserved in some members of the hematopoietin receptor superfamily. Numbers correspond to amino acid residues. Y, tyrosine.

repeats of the fibronectin type III module (Figure 1). The intracellular part of the human G-CSF-R consists of 183 amino acids. Several stretches of amino acids in the G-CSF-R cytoplasmic domain show limited sequence homology to other members of the hematopoietin receptor superfamily. Two of these stretches, referred to as box 1 and box 2, are positioned in the membrane-proximal region of G-CSF-R. Box 1 and box 2 are also present in several other hematopoietin receptors, e.g. EPO receptor, gp130, and the β chains of IL-2 and IL-3 receptors (20-24). The 53 membrane-proximal cytoplasmic amino acids of G-CSF-R containing the box 1 and box 2 motifs are indispensable for transduction of growth signals (24-26). Site-directed mutagenesis of G-CSF-R confirmed the importance of box 1 and box 2 in proliferative signaling. Substitution of the conserved prolines by alanines at positions 639 and 641 within box 1, as well as truncation of G-CSF-R between box 1 and box 2, completely abolished growth signal transduction by G-CSF-R (26-28). The next segment of 30 to 35 amino acids distal to box 2 contains a domain that enhances proliferative signaling in myeloid cells (25). The carboxy (C)-terminal (membrane-distal) region of G-CSF-R contains a third conserved motif, termed box 3, that is shared only with gp130 (23,24). This C-terminal region of approximately 100 amino acids is essential for transduction of neutrophilic differentiation signals (25,27). Deletion of the C-terminus of G-CSF-R not only destroys the differentiation signaling capacity but also removes a negative regulatory element inhibiting proliferative signaling (25). Experiments with a panel of G-CSF-R deletion mutants and chimeras of growth hormone receptor and G-CSF-R indicated that mutations in the membrane-proximal cytoplasmic region could also result in the abrogation of differentiation induction by G-CSF (27). These data demonstrate that differentiation signals from the C-terminal region of G-CSF-R cannot be given without the functional integrity of the membrane-proximal region.

1.5 G-CSF receptor: isoforms

Most hematopoietin receptor genes code for variant receptor proteins by alternative RNA splicing. Five different isoforms of human G-CSF-R have been identified that are all identical in the extracellular domain but differ in their downstream sequences (17,19,29). One form encodes a soluble receptor (pHQ2 or class II) that has only been found to be expressed in a leukemic cell line, U937 (17). The other isoforms differ in their cytoplasmic sequences and encode proteins of variable sizes. The most abundantly expressed (wild-type) G-CSF-R protein (class I) has a cytoplasmic domain of 183 amino acids, whereas the pHG11 receptor (class III) contains an insertion of 27 amino acids in the region between box 1 and box 2. The D7 receptor (class IV) lacks the 87 C-terminal amino acids and contains a distinct C-terminus of 34 amino acids. The SD receptor (class V) contains an altered C-terminus that is identical to D7 but truncated distal to box 2. Both D7 and SD receptor forms are expressed in normal human granulocytes, but at significantly lower

levels than the class I G-CSF-R isoform. The physiological significance of the alternative receptor proteins and the regulation of their expression are not yet clear. It is conceivable that some of them, in particular the soluble receptor forms, act as antagonists of growth factor responses. Notably, G-CSF-R forms with altered C-termini may be specifically hampered in transducing maturation signals (25).

1.6 G-CSF receptor: disease

Severe congenital neutropenia (SCN) and acute myeloblastic leukemia (AML) are diseases that are both characterized by a differentiation arrest in the myeloid lineage. Although originally described as an autosomal recessive entity, SCN comprises a heterogeneous group of disorders with variable inheritance, characterized by recurrent bacterial infections and severe absolute neutropenia usually presented early in infancy. Most SCN patients respond favorably to *in vivo* G-CSF administration with clinical improvement and significant increases in circulating neutrophils, whereas GM-CSF is generally ineffective in stimulating neutrophil production in these patients. Although normal to increased numbers of G-CSF-R have been detected on neutrophils from SCN patients with normal ligand binding affinity, cultured marrow cells from SCN patients frequently show a reduced responsiveness to G-CSF. In 1994, a case of SCN was reported with an acquired mutation in the *G-CSF-R* gene (30). This mutation introduced a premature stop codon resulting in truncation of 98 C-terminal amino acids of the G-CSF-R protein. As expected, upon its ectopic expression in murine myeloid cell lines, the truncated G-CSF-R from the SCN patient failed to transduce differentiation signals. The mutation was present in the myeloid cells of the patient, but not in erythroid cells, T and B lymphocytes, and skin fibroblasts. These findings indicate that progenitor cells committed to the myeloid lineage had been the target cell population for the acquired mutation and had clonally expanded. In addition, similar mutations in the *G-CSF-R* gene have been detected in 15 to 20% of SCN patients, all leading to C-terminal truncations of the differentiation-inducing domain of G-CSF-R (31,32). Recently, it was shown that these G-CSF-R mutations also cause neutropenia in a 'knock-in' mouse model, providing further *in vivo* evidence that the C-terminal region of G-CSF-R is essential for normal neutrophil production (33). Notably, SCN patients have an increased probability of developing AML, which suggests that the same or functionally related defects may be involved in the pathogenesis of both diseases (34). Patients with SCN harboring G-CSF-R mutations may represent a subgroup of patients in whom the neutropenia is a preleukemic disorder. In support of this, several AML patients with a history of SCN have been reported with very similar mutations in the *G-CSF-R* gene, again truncating the C-terminal differentiation-inducing region (31,32).

Another G-CSF-R mutation associated with leukemia was found in the leukemic blasts of a patient with *de novo* AML (29). The AML blasts of this patient showed high expression of the SD splice variant (class V), in which the C-terminus was

altered due to a change in the reading frame, whereas expression of this G-CSF-R isoform is low in normal granulocytes. Analysis of *G-CSF-R* genomic DNA isolated from the AML cells revealed a G to A transition next to the (cryptic) splice donor site involved in the alternative RNA splicing. The SD receptor appeared to be unable to transduce both proliferation and differentiation signals upon transfer to factor dependent cell lines. In agreement with this, the AML cells of the patient also failed to respond to G-CSF in proliferation assays, whereas high responsiveness to either IL-3 or GM-CSF was noted. It was postulated that myeloid cells from this patient overexpressing the nonfunctional G-CSF-R isoform lacked the capacity to terminally differentiate in response to G-CSF, but could survive and proliferate in response to other available HGFs *in vivo*.

These findings indicate that mutations in the C-terminal region of G-CSF-R, specifically affecting the differentiation signaling function of the receptor, are sometimes involved in the pathogenesis of SCN and AML. They further imply that genes encoding signaling molecules that are specifically activated through the C-terminal domain of G-CSF-R are additional potential targets for transforming mutations leading to AML. Identification of these genes and study of the signaling properties of their products in normal and leukemic cells are expected to elucidate the contribution of genetic defects, specifically interfering with G-CSF-induced neutrophilic differentiation, in the development of leukemia.

1.7 G-CSF receptor: signal transduction

A general first step in the signaling processes of HGF is ligand-induced dimerization or oligomerization of receptor components whose cytoplasmic regions interact to initiate a downstream signaling cascade. Some HGF receptor systems are composed of heteromeric complexes, comprising two or three different receptor chains. Other receptor structures, like G-CSF-R, form homodimeric complexes upon ligand binding. Although members of the hematopoietin receptor superfamily lack intrinsic tyrosine kinase activity, they activate cytoplasmic tyrosine kinases, in particular of the Janus kinase (Jak) family (35-37). Jaks associate with the membrane-proximal cytoplasmic region of the receptors and become activated upon ligand-induced receptor dimerization or oligomerization (29,38-40). Mutations in the membrane-proximal cytoplasmic region that prevent Jak binding and activation completely abolish the proliferative signaling capacities of several receptor systems (39,41). Thus, activation of Jaks correlates with the mitogenic signaling function of HGF receptors. In support of this, overexpression of a kinase-deficient form of Jak2 interferes with EPO-induced proliferation (42).

Upon their activation, presumably involving autophosphorylation on tyrosines, Jaks induce phosphorylation of the STAT (signal transducer and activator of transcription) proteins on a conserved tyrosine residue, just C-terminal to the Src homology 2 (SH2) domain (43). Subsequently, STATs dimerize by

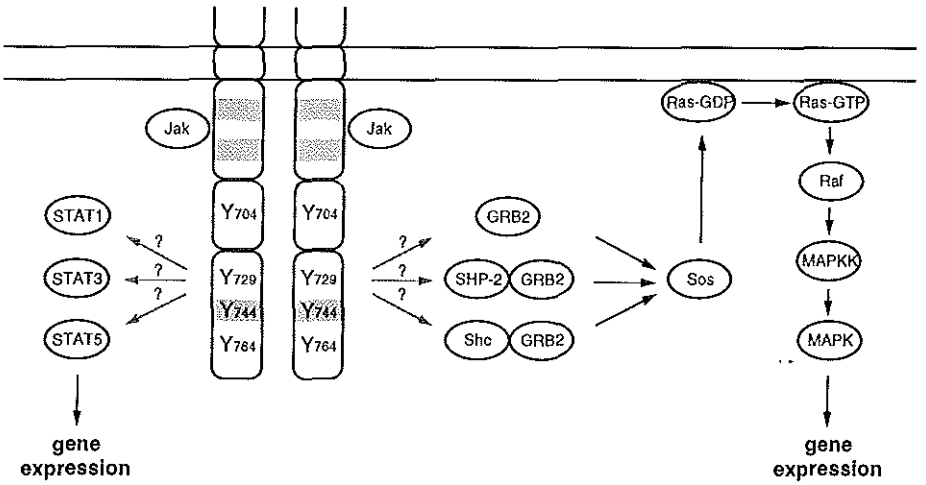

Figure 2. A model for signal transduction pathways activated by G-CSF-R. Ligand-induced homodimerization of G-CSF-R results in Jak activation and phosphorylation of the cytoplasmic tyrosine residues of G-CSF-R. These phosphorylated tyrosines form potential binding sites for SH2 domain-containing signaling molecules, such as STAT proteins, Shc, SHP-2, and GRB2. After binding, most of these molecules are tyrosine-phosphorylated themselves. Phosphorylated STATs dimerize, translocate to the nucleus, and induce gene expression. Phosphorylated Shc and SHP-2 interact with GRB2. Subsequently, GRB2 binds to Sos, leading to $p21^{Ras}$ activation and ultimately to induction of transcription.

phosphotyrosine/SH2 interactions, translocate to the nucleus and activate target genes by interaction with specific DNA sequences. G-CSF stimulation results in rapid activation of Jak1, Jak2, STAT1, STAT3, and STAT5 (Figure 2) (29,44-46). Recently, a role for STATs in the control of cell growth and development has been demonstrated in a variety of cell systems. For instance, it was shown that STAT5 is involved in IL-3-induced proliferation (47). Activation of STAT3 was linked to IL-6-induced growth arrest and macrophage differentiation (48), IL-10-induced inhibition of macrophage proliferation (49), and G-CSF-induced neutrophilic differentiation (50). However, the underlying mechanism(s) of STAT-mediated control of growth and differentiation are still largely unclear.

Two other cytoplasmic tyrosine kinases involved in G-CSF signaling are Lyn and Syk (51). Lyn appears to be constitutively associated with the G-CSF-R cytoplasmic domain, whereas Syk is recruited into the G-CSF-R/Lyn complex following activation of G-CSF-R. By using G-CSF-R expressing transfectants of $Lyn^{-/-}$ and $Syk^{-/-}$ chicken B cell lines, it was shown that Lyn but not Syk is essential for G-CSF-R-mediated proliferative signaling (52). $Syk^{-/-}$ knockout mice have normal neutrophil levels, and bone marrow cells from these animals respond normally to

G-CSF in *in vitro* colony cultures, further suggesting that Syk does not contribute in a major way to G-CSF-driven granulopoiesis (53).

Tyrosine kinase activity induced by G-CSF also results in the rapid phosphorylation of tyrosine residues of the G-CSF-R protein itself, although it is still unknown which kinases mediate this process (44,54). The cytoplasmic domain of G-CSF-R contains four conserved tyrosines, at positions 704, 729, 744, and 764 (Figure 1). Phosphorylated tyrosine residues form potential binding sites for signaling molecules that contain SH2 domains (55). The specificity of binding via these domains is largely determined by the amino acids directly flanking the phosphorylated tyrosine residue (56). For instance, STAT3 proteins are recruited to the cytoplasmic domains of gp130 and LIF receptor via interaction of the SH2 domain of STAT3 with receptor tyrosine residues that are present in a tyrosine-X-X-glutamine (YXXQ) sequence (57).

Another major signal transduction route that involves SH2 domain-containing proteins is the $p21^{Ras}$ pathway (Figure 2). The signaling molecules Shc, SHP-2 (Syp; a protein tyrosine phosphatase), and GRB2 function as adapter proteins in this pathway by linking phosphorylated receptors to downstream effectors. After activation of a variety of receptors, Shc and SHP-2 are tyrosine-phosphorylated, providing binding sites for the SH2 domain of GRB2. Subsequently, GRB2 can bind to the $p21^{Ras}$ guanine nucleotide exchange factor, Son of sevenless (Sos) (58,59). Thus, translocation of Sos to the plasma membrane, that leads to activation of $p21^{Ras}$, can be mediated either by direct binding of GRB2 to the phosphorylated receptor or via involvement of tyrosine-phosphorylated Shc or SHP-2 (60-64). Activation of $p21^{Ras}$ is achieved by converting this molecule from the GDP-bound form to the GTP-bound form. $p21^{Ras}$-GTP triggers activation of a cascade of downstream protein kinases including serine/threonine kinase Raf, dual-specificity kinase MAP (mitogen-activated protein) kinase kinase, and the serine/threonine kinase MAP kinase, eventually leading to induction of gene expression. G-CSF, like other HGFs such as IL-2, IL-3, GM-CSF, and EPO, activates the $p21^{Ras}$ signal transduction pathway (65-67). Previous studies provided evidence for the involvement of $p21^{Ras}$ in IL-3- and G-CSF-induced cell cycle progression. Expression of a dominant-inhibitory mutant of $p21^{Ras}$ in myeloid cells interferes with IL-3- and G-CSF-mediated proliferation and causes a G1 arrest (68). In contrast, G-CSF still induces neutrophilic differentiation in these cells, suggesting that activation of the $p21^{Ras}$ pathway is not a prerequisite for differentiation induction. It is, however, unknown which regions of the cytoplasmic domain of G-CSF-R are important for $p21^{Ras}$ activation, and which mechanisms connect G-CSF-R to this signaling pathway.

1.8 Scope of this thesis

G-CSF-R transduces signals that are involved in proliferation, differentiation, and survival of myeloid cells. The presence of discrete functional regions in the cytoplasmic domain of G-CSF-R suggests the existence of multiple signaling pathways that are activated via these regions to elicit distinct biological effects. Signal transduction by G-CSF-R, however, is still poorly understood. Insight into the structure/function features of G-CSF-R is not only important for understanding the signaling mechanisms, but may also clarify the pathogenesis of diseases characterized by defective granulopoiesis, such as SCN and AML.

Experiments described in Chapter 2 were performed to identify the signaling intermediates of the $p21^{Ras}$ pathway that are activated by G-CSF-R. The ability of wild-type, C-terminal deletion mutants, and tyrosine-to-phenylalanine substitution mutants of G-CSF-R to activate Shc- and/or GRB2-containing complexes was examined. In Chapter 3, the same G-CSF-R constructs were used to determine which cytoplasmic regions of G-CSF-R are involved in activation of STAT1 and STAT3. The role of Jak kinases in activation of the $p21^{Ras}$ pathway was investigated by constructing a mutant in which tryptophan 650 in the membrane-proximal region of G-CSF-R was replaced by arginine (Chapter 4). This mutant, that appeared to be unable to activate Jaks, was tested for its capacity to activate signaling proteins of the $p21^{Ras}$ pathway. In Chapter 5, tyrosine-to-phenylalanine substitution mutants of G-CSF-R were expressed in differentiation competent myeloid cells to determine the role of the cytoplasmic tyrosine residues in G-CSF-mediated responses. Finally, the involvement of STAT3 in G-CSF-induced proliferation and neutrophilic differentiation was examined by overexpressing dominant-negative STAT3 mutants in differentiation competent myeloid cells (Chapter 6).

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CHAPTER 2

Specific involvement of tyrosine 764 of human granulocyte colony-stimulating factor receptor in signal transduction mediated by p145/Shc/GRB2 or p90/GRB2 complexes

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Abstract

Signal transduction from the granulocyte colony-stimulating factor receptor (G-CSF-R) occurs via multiple pathways, one of which involves activation of $p21^{Ras}$ and mitogen-activated protein kinase. The SH2 domain-containing proteins Shc and GRB2 have been implicated in this latter signaling route. We studied the role of these proteins in signal transduction from wild-type (WT) G-CSF-R, C-terminal deletion mutants, and tyrosine-to-phenylalanine substitution mutants in transfectants of the mouse pro-B cell line, BAF3. G-CSF stimulation of BAF3 cells expressing WT G-CSF-R induced tyrosine phosphorylation of Shc. Anti-Shc antibodies co-immunoprecipitated tyrosine-phosphorylated 145-kD proteins (p145), whereas GRB2 immunoprecipitates contained phosphorylated Shc, SHP-2 (Syp), and proteins of 145 and 90 kD (p90). Neither of these complexes were detected after activation of a C-terminal deletion mutant of G-CSF-R that lacked all four conserved cytoplasmic tyrosine residues. G-CSF induced formation of SHP-2/GRB2 complexes in all the tyrosine-substitution mutants, suggesting that this association did not depend on the presence of single specific tyrosine residues in G-CSF-R. In contrast, tyrosine 764 of G-CSF-R appeared to be exclusively required for tyrosine phosphorylation of Shc and its association with p145 and GRB2. In addition, tyrosine 764 also specifically mediated binding of GRB2 to p90 without the involvement of Shc. These findings indicate that tyrosine 764 of G-CSF-R has a prominent role in G-CSF signal transduction.

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a 20- to 25-kD glycoprotein secreted by bone marrow stroma cells, macrophages, fibroblasts, and endothelial cells. G-CSF plays an essential role in the regulation of granulopoiesis and the maintenance of neutrophil levels in the peripheral blood. G-CSF stimulates proliferation, survival, and maturation of cells committed to the neutrophilic granulocyte lineage (1,2). The diverse biological effects of G-CSF are mediated through a single class of cell-surface receptor proteins that form homodimeric complexes upon ligand binding. The human G-CSF receptor (G-CSF-R) is a member of the cytokine receptor superfamily (3,4). Although intracellular domains of cytokine receptors show little homology, certain similarities have been reported. For instance, the cytoplasmic membrane-proximal region of G-CSF-R, erythropoietin receptor (EPO-R), interleukin (IL)-6 signal transducer gp130, and the β chains of IL-2 and IL-3 receptors contains two conserved subdomains known as box 1 and box 2. This region is indispensable for transduction of growth signals (5-11). Importantly, the carboxy-terminal region of the cytoplasmic domain of G-CSF-R appears to be predominantly involved in the induction of neutrophilic maturation (9,11).

Cytokine receptors lack intrinsic tyrosine kinase activity themselves, but activate

cytoplasmic tyrosine kinases (2,4,12). Recent studies have shown that binding of G-CSF to its receptor results in rapid phosphorylation of tyrosines of the receptor protein itself (13,14) and of the Janus kinases, Jak1 (14,15) and Jak2 (15,16). The cytoplasmic tyrosine kinases, Lyn and Syk, have also been suggested to play a role in G-CSF-R signal transduction (17).

Phosphorylated tyrosine residues form potential binding sites for cellular Src homology 2 (SH2) domain-containing signaling molecules (18). The specificity of these interactions is determined by the abilities of different SH2 domains to recognize different sequences surrounding the phosphorylated tyrosine (19,20). SH2 domains have been identified in a wide variety of molecules, including those with enzymatic activity, such as Src family tyrosine kinases, $p21^{Ras}$ GTPase activating protein (21), phospholipase Cγ (22), phosphotyrosine phosphatases (HCP/PTP1C and SHP-2/Syp/PTP1D) (23-25), and Vav (26), and those with no obvious enzymatic activity, including Shc (27), GRB2 (28), Crk (29), and Nck (30). The SH2 domain-containing proteins with no apparent enzymatic activity are believed to act as adapter molecules, linking phosphorylated receptors to downstream effectors. For instance, Shc and GRB2 function as adapter proteins in the $p21^{Ras}$/mitogen-activated protein (MAP) kinase signal transduction pathway (31,32). Shc is tyrosine-phosphorylated after activation of a variety of receptors, providing a binding site for the SH2 domain of GRB2 (33). GRB2 can be bound to the $p21^{Ras}$ guanine nucleotide exchange factor, Son of sevenless (Sos) (34,35). Translocation of Sos to the plasma membrane, leading

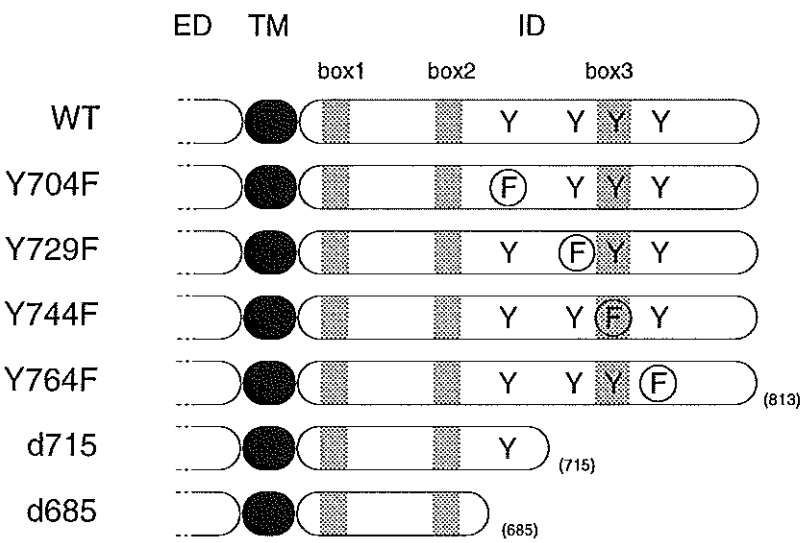

Figure 1. Schematic diagram of G-CSF-R proteins. Boxes 1, 2, and 3 denote subdomains conserved in some members of the cytokine receptor superfamily. Numbers in parentheses mark amino acid positions. ED, extracellular domain; TM, transmembrane domain; ID, intracellular domain; Y, tyrosine; F, phenylalanine.

to activation of $p21^{Ras}$, can be mediated either by direct binding of GRB2 to the phosphorylated receptor (28,36) or via involvement of tyrosine-phosphorylated Shc (33,37) or SHP-2 (36,38).

The human G-CSF-R cytoplasmic domain contains 4 tyrosine residues, located at positions 704, 729, 744, and 764. To establish the role of each of these tyrosines in G-CSF signaling, we individually replaced these residues with phenylalanine using a polymerase chain reaction (PCR)-based mutation strategy. We show that activation of wild-type (WT) G-CSF-R induces rapid tyrosine phosphorylation of Shc and association of Shc with GRB2. Tyrosine 764 of G-CSF-R appears to be indispensable for G-CSF-induced phosphorylation of Shc. Activation of Shc is accompanied by tyrosine phosphorylation and association of 145-kD proteins of unknown identity, resulting in the formation of p145/Shc/GRB2 complexes. Tyrosine 764 is also predominantly involved in the phosphorylation of 90-kD proteins that form complexes with GRB2. Finally, we demonstrate that the C-terminal domain of activated G-CSF-R mediates binding of GRB2 to tyrosine-phosphorylated SHP-2. In contrast to the formation of p145/Shc/GRB2 or p90/GRB2 complexes, SHP-2/GRB2 binding is not mainly accomplished via one tyrosine residue of G-CSF-R.

Materials and methods

Site-directed mutagenesis. Human *G-CSF-R* cDNA was cloned in the pBluescript vector (pBS) and in the eukaryotic expression vector pLNCX (39), as described previously (11). PCR techniques were used to individually substitute the four tyrosine residues located in the cytoplasmic domain of G-CSF-R for phenylalanine residues (Figure 1), according to a recently described method for site-directed mutagenesis (40). The oligonucleotides 5' CAGACCTTTGTGCTGCAGGG 3' (Y704F, containing a silent *Pst*I site), 5' GATCAAGTACTTTTTGGGCAGCT 3' (Y729F, containing a silent *Sca*I site), 5' GGGCACTTTCTCCGCTGTGAC 3' (Y744F), and 5' AGCCCCAAAAGCTTTGAGAACCT 3' (Y764F, containing a silent *Hin*dIII site) were designed as mutagenic primers. The human *G-CSF-R* cDNA cloned in pBS was used as the template in the first PCR, with a mutagenic primer and with primer M13-20 (5'-GTAAAACGACGGCCAGT-3'), a universal primer specific for pBS. In the second PCR, the human *G-CSF-R* cDNA cloned in pLNCX was used as the template, with the product of the first PCR as a megaprimer and with primers M13-20 and FR2 (5'-TGTGATCATCGTGACTCCCTT-3'). The PCR products were cloned in pLNCX. All mutations were confirmed by nucleotide sequencing. Deletion mutants d715 (DA) and d685 (M1) have been described previously (11).

Cells and transfection. The IL-3-dependent murine pro-B cell line, BAF3 (41), was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 ng/ml of murine IL-3. Transfection of BAF3 cells was performed by electroporation. Following gene transfer, cells were cultured in IL-3-containing medium for 24 hours and then selected in G418 (Gibco-BRL, Breda, The

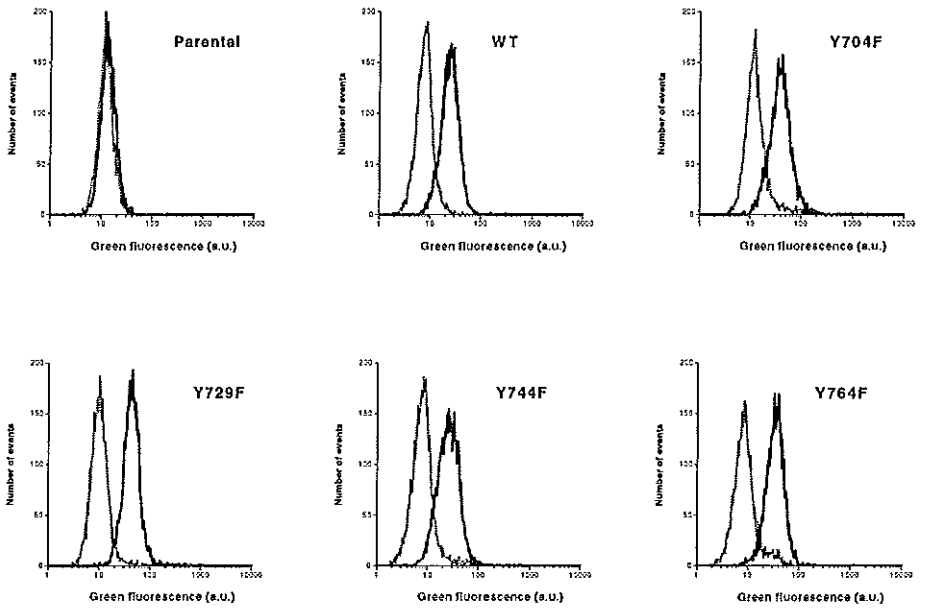

Figure 2. Flow cytometric analysis of G-CSF-R expression on parental BAF3 cells and BAF3 transfectants. Cells were stained either with rabbit anti-human G-CSF-R antibodies followed by fluorescein-conjugated goat-anti-rabbit IgG (black lines) or with fluorescein-conjugated goat-anti-rabbit IgG alone (grey lines). a.u., arbitrary units.

Netherlands) at a concentration of 1.5 mg/ml. Multiple clones were expanded for further analysis. Reverse transcriptase-PCR using the primers FRM1 (5' CTGCTGTTGTTAACCTGCCTC 3') and RV25.1 (5' GTAGATCTTAGTCATGG-GCTTATGG 3') was performed to check the proper expression of WT and mutant G-CSF-R constructs. The products were either digested by *PstI* (Y704F), *ScaI* (Y729F), and *Hin*dIII (Y764F) or sequenced following cloning in pBS (Y744F). To detect G-CSF-R proteins on the cell surface, cells were treated for 30 minutes at 4°C with rabbit anti-human G-CSF-R antibody 1729 (1:100 dilution; raised against amino acids 17 to 344 in the extracellular domain of the receptor). After washing, cells were incubated for 30 minutes at 4°C with a 1:40 dilution of fluorescein-conjugated goat-anti-rabbit IgG (Nordic Immunology, Tilburg, The Netherlands). Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA).

DNA synthesis assay. DNA synthesis was assayed by [^{3}H]-thymidine (^{3}H-TdR) uptake, as described previously (11). Briefly, cells (1 x 10^4) were incubated in triplicate in 100 µl 10% fetal calf serum-RPMI medium supplemented with titrated concentrations of human G-CSF or murine IL-3 in 96-well plates for 48 hours. Eight hours before cell harvesting, 0.1 µCi ^{3}H-TdR (2 Ci/mM; Amersham International, Amersham, UK) was added to each well. ^{3}H-TdR incorporation was measured by liquid scintillation counting.

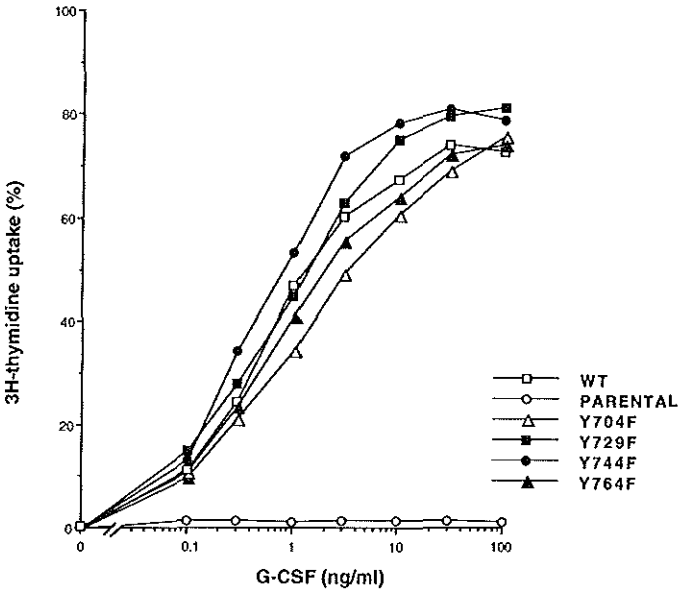

Figure 3. G-CSF responses of parental BAF3 cells and BAF3 transfectants in ^{3}H-TdR uptake assays. Data are expressed as percentages of maximal response to murine IL-3.

Preparation of cell lysates, immunoprecipitation, and Western blotting. Before stimulation, cells were deprived of serum and factors for 10 hours at 37°C in RPMI 1640 medium. One hour before addition of growth factors, 10 μM sodium orthovanadate (Na_3VO_4) was added. Cells (5 x 10^6/ml) were incubated in RPMI 1640 medium in the presence of human G-CSF (100 ng/ml) or murine IL-3 (1 μg/ml) for 1 to 30 minutes at 37°C or without factors (control). At the different time points, 10 vol ice-cold phosphate-buffered saline and 10 μM Na_3VO_4 were added. Subsequently, cells were centrifuged and lysed by incubation for 1 hour at 4°C in lysis buffer (20 mM Tris hydrochloride [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, 50 μg/ml iodoacetamide). Insoluble materials were removed by centrifugation for 30 minutes at 10,000*g* at 4°C. Immunoprecipitations were performed essentially as previously described (16). In brief, supernatants of the clarified cell lysates were incubated overnight at 4°C with either anti-Shc (37) or anti-GRB2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA) antibodies. Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) were then added for 1 hour at 4°C. After washing the beads with ice-cold lysis buffer five times, bound proteins were eluted by boiling for 5 minutes in sodium dodecyl sulphate (SDS) sample buffer. Following SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were electroblotted onto nitrocellulose (0.2 μm; Schleicher & Schuell, Dassel, Germany). Filters were blocked by incubation in 0.3% Tween 20 in

Tris-buffered saline ([TBS] 10 mM Tris hydrochloride [pH 7.4], and 150 mM NaCl) for 1 hour at 37°C, washed in TBST (0.05% Tween 20 in TBS), and incubated with antibodies diluted in TBST. Antibodies used for Western blotting were antiphosphotyrosine antibody 4G10 (Upstate Biotechnology Inc, Lake Placid, NY), anti-Shc, anti-GRB2, and anti-SHP-2 (Santa Cruz). 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 hydrochloride [pH 6.7], 2% SDS, and 100 mM β-mercaptoethanol at 50°C for 30 minutes, reblocked, washed, and reprobed.

Glutathione S-transferase/GRB2 capture. Cell lysates, prepared as described earlier, were incubated for 2 hours at 4°C with 2 μg glutathione S-transferase (GST)/GRB2 fusion protein (37) bound to glutathione-Sepharose beads (Pharmacia). After washing the loaded beads in ice-cold lysis buffer five times, bound proteins were eluted by boiling for 5 minutes in SDS sample buffer, separated by SDS-PAGE, and analyzed by Western blotting, as described earlier.

Results

Expression of and mitogenic signaling by different forms of G-CSF-R. To determine expression levels of mutated G-CSF-R proteins as compared with WT proteins in BAF3 transfectants, cells were stained with anti-human G-CSF-R antibodies and analyzed by flow cytometry. Examples of clones expressing WT or mutant G-CSF-R proteins are shown in Figure 2. The abilities of transfectants to proliferate in response to G-CSF were examined in ^{3}H-TdR uptake assays. G-CSF responses of BAF3 cells expressing the tyrosine-to-phenylalanine substitution mutants were comparable to responses of BAF3 cells expressing WT G-CSF-R (Figure 3). Repeated analyses of at least three independent clones of each mutant gave identical results. G-CSF-R levels and G-CSF responses of BAF3 cells expressing deletion mutants d685 (M1) and d715 (DA) have been described previously and were essentially similar to those of BAF3 cells expressing WT G-CSF-R (11).

G-CSF induces tyrosine phosphorylation of Shc. To investigate whether Shc is tyrosine-phosphorylated after G-CSF treatment, BAF3 cells expressing WT G-CSF-R were stimulated with G-CSF for 1 to 30 minutes. For comparison, cells were incubated in parallel without growth factor or with IL-3. IL-3 has previously been shown to induce tyrosine phosphorylation of Shc (42,43). Phosphorylation of Shc was induced following stimulation with G-CSF and IL-3 (Figure 4A). Of three Shc isoforms, i.e. p46^{Shc}, p52^{Shc}, and p66^{Shc}, that have been described (27), p52^{Shc} was predominantly phosphorylated. Only minimal activation of p46^{Shc} was seen, whereas p66^{Shc} is not present at detectable levels in BAF3 cells (Figure 4C). Upon stimulation with G-CSF, tyrosine phosphorylation of Shc was induced within 1 minute, reached a maximum at 5 minutes, and declined after 10 minutes. Tyrosine-phosphorylated

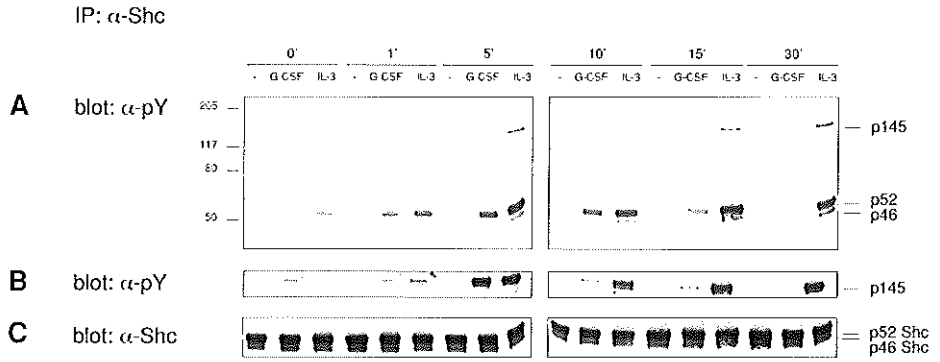

Figure 4. Shc immunoprecipitation on lysates from BAF3 cells expressing WT G-CSF-R. Serum- and growth factor-deprived cells were incubated at 37°C without factor (-), with G-CSF (100 ng/ml), or with IL-3 (1 µg/ml) for the times indicated. (A) Western blot hybridized with antiphosphotyrosine antibody, 4G10. (B) Longer exposure of part of A. (C) Blot reprobed with anti-Shc antibodies to confirm equal loading of Shc. Positions of molecular mass standards (in kD) are indicated.

proteins of approximately 145 kD (p145) co-immunoprecipitated with tyrosine-phosphorylated Shc after G-CSF and IL-3 treatment (Figures 4A and B). Reprobing of the blot with anti-Shc antibodies confirmed equal loading of the samples (Figure 4C) and showed that the 145-kD proteins were not recognized on Western blots by anti-Shc antibodies, and thus co-precipitated by virtue of their association with Shc (data not shown).

Tyrosine 764 of G-CSF-R is exclusively involved in activation of Shc. To determine which of the tyrosine residues located in the cytoplasmic domain of G-CSF-R are involved in activation of Shc, BAF3 transfectants expressing C-terminal deletion or substitution mutants of G-CSF-R (Figure 1) were studied. G-CSF-

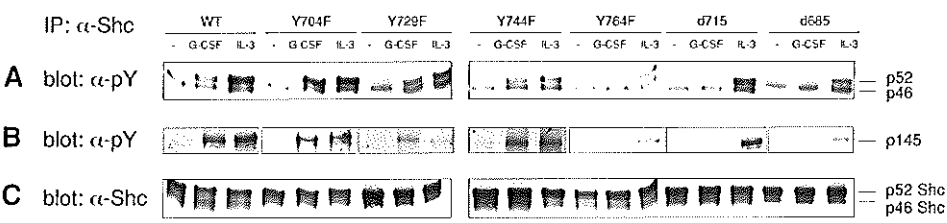

Figure 5. Shc immunoprecipitation on lysates from BAF3 cells expressing mutant G-CSF-R proteins. Serum- and growth factor-deprived cells were incubated for 5 minutes at 37°C without factor (-), with G-CSF, or with IL-3. (A and B) Western blot hybridized with antiphosphotyrosine antibody, 4G10. (C) Blot reprobed with anti-Shc antibodies to confirm equal loading of Shc.

stimulated tyrosine phosphorylation of Shc and its associated p145 was observed in cells expressing WT G-CSF-R and substitution mutants Y704F, Y729F, and Y744F (Figures 5A and B). In contrast, no activation of Shc and p145 was seen after G-CSF stimulation in BAF3 transfectants expressing substitution mutant Y764F and deletion mutants d685 and d715, despite the equal presence of Shc proteins as compared with WT G-CSF-R transfectants (Figure 5C). In parallel control incubations, IL-3 induced tyrosine phosphorylation of Shc and p145 in all of these transfectants. Repeated analyses of at least three independent clones of each mutant gave identical results.

G-CSF induces association of GRB2 with tyrosine-phosphorylated Shc, SHP-2, 90-kD, and 145-kD proteins. Anti-GRB2 immunoprecipitates from unstimulated and G-CSF- or IL-3-stimulated BAF3 cells expressing WT G-CSF-R were probed with anti-PY antibodies (Figure 6A). Following incubation with G-CSF and IL-3, tyrosine-phosphorylated proteins of approximately 52, 70, 90, and 145 kD co-precipitated with GRB2. The kinetics of G-CSF-stimulated association of GRB2 with tyrosine-phosphorylated 52- and 145-kD proteins were comparable to the kinetics of tyrosine phosphorylation of Shc and its association with p145, as seen in anti-Shc

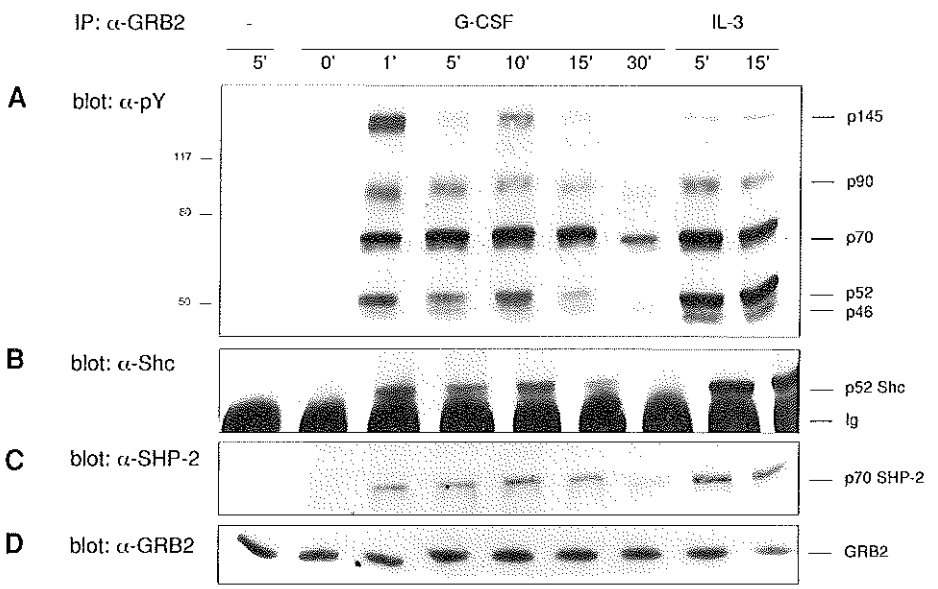

Figure 6. GRB2 immunoprecipitation on lysates from BAF3 cells expressing WT G-CSF-R. Cells were treated as indicated in Figure 4. (A) Phosphotyrosine-containing proteins identified by Western blotting with 4G10. (B) Blot reprobed with anti-Shc antibodies. Ig, rabbit immunoglobulins. (C) Blot reprobed with anti-SHP-2 antibodies. The p70 SHP-2 band in panel C matched the upper part of the p70 band in panel A. (D) GRB2 immunoprecipitates separated on 12% polyacrylamide and probed with anti-GRB2 antibodies to confirm equal loading of GRB2.

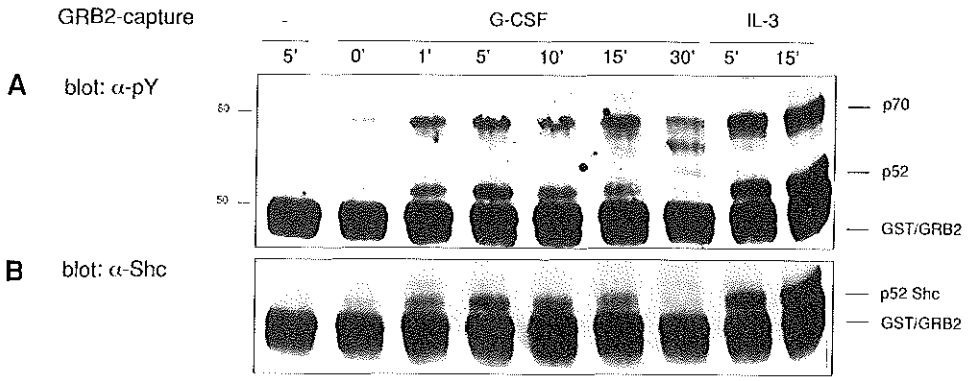

Figure 7. Association of GST/GRB2 fusion proteins with tyrosine-phosphorylated proteins. Lysates from BAF3 cells expressing WT G-CSF-R, treated as indicated in Figure 4, were incubated for 2 hours at 4°C with GST/GRB2 fusion proteins immobilized on glutathione-Sepharose beads. Bound proteins were eluted and subjected to SDS-PAGE. (A) Phosphotyrosine-containing proteins identified by Western blotting with 4G10. (B) Blot reprobed with anti-Shc antibodies.

immunoprecipitates (Figures 4A and B). The somewhat lower reactivity of p145 with anti-PY antibodies observed after 5 minutes of stimulation with G-CSF was not confirmed in repeated experiments. Reprobing of the blot with anti-Shc antibodies identified the 52-kD protein as Shc (Figure 6B). Upon G-CSF stimulation, tyrosine phosphorylation of 70-kD proteins occurred within 1 minute and persisted for at least 30 minutes. Reprobing the blot revealed that a subfraction of the 70-kD proteins reacted with anti-SHP-2 antibodies (Figure 6C). Essentially similar results were obtained using GST/GRB2 fusion proteins (Figure 7).

Mutation Y764F reduces G-CSF-induced association of GRB2 with tyrosine-phosphorylated Shc, 90-kD, and 145-kD proteins. Since G-CSF-R mutant Y764F failed to induce tyrosine phosphorylation of Shc and since GRB2 only associates with Shc in its tyrosine-phosphorylated form, anti-GRB2 immunoprecipitates from G-CSF-stimulated BAF3 cells expressing mutant Y764F should not contain Shc. As shown in Figure 8A, G-CSF stimulation only induced association of GRB2 with tyrosine-phosphorylated 70-kD proteins that partly hybridized with anti-SHP-2 antibodies after reprobing (data not shown). The kinetics correspond to the kinetics seen in BAF3 cells expressing WT G-CSF-R. G-CSF stimulation did not induce association of GRB2 with phosphorylated Shc or with proteins of 145 kD, whereas in parallel control incubations, IL-3 stimulation did (Figures 8A and B). Although in the exposure presented the 145-kD proteins are not clearly visible after IL-3 stimulation, they were visible in longer exposures of the same blot (data not shown). In contrast to WT G-CSF-R, activation of mutant Y764F only marginally induced association of GRB2 with phosphorylated 90-kD proteins.

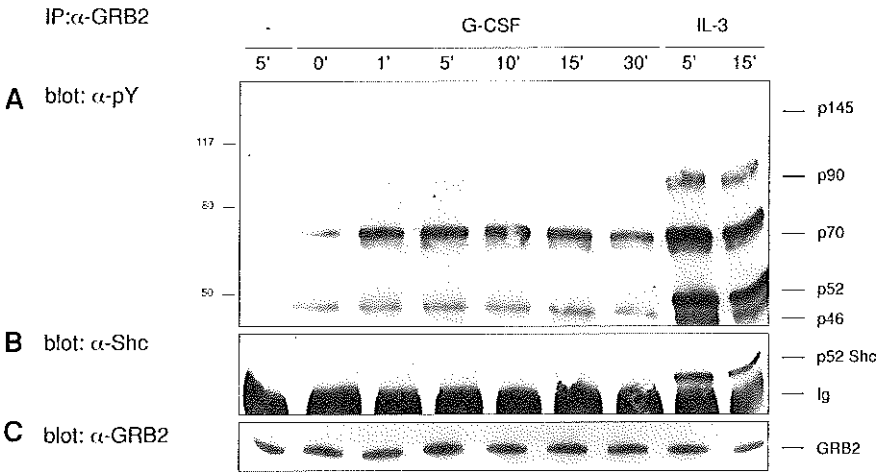

Figure 8. GRB2 immunoprecipitation on lysates from BAF3 cells expressing G-CSF-R mutant Y764F. Cells were treated as indicated in Figure 4. (A) Phosphotyrosine-containing proteins identified by Western blotting with 4G10. (B) Blot reprobed with anti-Shc antibodies. (C) Blot probed with anti-GRB2 antibodies (see Figure 6D).

The C-terminal region of G-CSF-R is required for G-CSF-stimulated association of GRB2 with tyrosine-phosphorylated SHP-2. To determine whether the C-terminal region of G-CSF-R is involved in association of GRB2 with tyrosine-phosphorylated SHP-2, BAF3 transfectants expressing deletion and substitution mutants of G-CSF-R were studied. As expected, G-CSF-stimulated association of GRB2 with tyrosine-phosphorylated Shc was observed in cells expressing substitution mutants Y704F, Y729F, and Y744F, but not in cells expressing deletion mutants d685 and d715 (Figure 9A). Tyrosine-phosphorylated 70- and 90-kD proteins were associated with GRB2 after G-CSF stimulation of cells expressing substitution

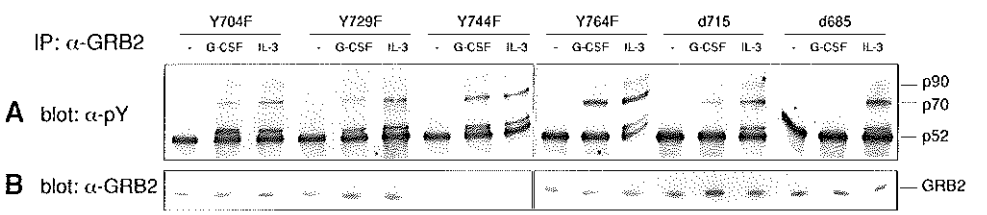

Figure 9. GRB2 immunoprecipitation on lysates from BAF3 cells expressing mutant G-CSF-R proteins. Cells were treated as indicated in Figure 5. (A) Phosphotyrosine-containing proteins identified by Western blotting with 4G10. (B) Blot probed with anti-GRB2 antibodies (see Figure 6D).

mutants Y704F, Y729F, and Y744F, but not deletion mutant d685. These associations were severely reduced in cells expressing deletion·mutant d715 (Figure 9A). After stripping and reprobing of the blot, part of the 70-kD proteins reacted with anti-SHP-2 antibodies (data not shown). Repeated analyses of at least three independent clones of each mutant gave identical results.

Discussion

Signaling from G-CSF-R involves at least two major routes, i.e. the Jak/STAT and $p21^{Ras}$/MAP kinase pathways (14-16,44). The membrane-proximal cytoplasmic regions of G-CSF-R, EPO-R, and other members of the cytokine receptor family have been shown to be primarily responsible for activation of Jak kinases (16,45,46). For some receptors, e.g. EPO-R and the β_c chain of IL-3-R and granulocyte-macrophage colony-stimulating factor (GM-CSF)-R, it was demonstrated that Jak proteins physically associate with the membrane-proximal region (45,47). In EPO-R, a single conserved tryptophan residue in this region is crucial for binding of Jak2 (48). So far, only limited information is available as to which regions or single amino acid residues within G-CSF-R are important for activation of $p21^{Ras}$ and MAP kinase and which mechanisms connect G-CSF-R to this signaling pathway. Shc and GRB2 proteins have been implicated in linking different types of cytokine receptors to $p21^{Ras}$ activation (42,43,49,50).

In this study, we established that activation of G-CSF-R results in rapid tyrosine phosphorylation of Shc and association of Shc with phosphorylated proteins of approximately 145 kD. Similar observations have been made for other cytokine receptors, including those for IL-3, EPO, GM-CSF, macrophage colony-stimulating factor (M-CSF), and Steel factor (43,50,51). The identity of the 145-kD proteins is not yet known. Recently, it was shown that Shc contains a novel phosphotyrosine-binding (PTB) domain, distinct from SH2 domains, which is involved in the interaction of Shc with tyrosine-phosphorylated 145-kD proteins (52).

GRB2 immunoprecipitates from G-CSF-activated BAF3 cell transfectants expressing WT G-CSF-R contained tyrosine-phosphorylated proteins of 52 (identified as Shc), 70, 90, and 145 kD. The kinetics of G-CSF-induced GRB2 association with Shc were comparable to those of tyrosine phosphorylation of Shc. Complex formation of 145-kD proteins, Shc, and GRB2 did not occur in cells expressing G-CSF-R deletion mutants d685 or d715 or substitution mutant Y764F, consistent with the notion that recruitment of these signaling molecules occurs exclusively via involvement of tyrosine 764 of G-CSF-R. Tyrosine 764 also determined G-CSF-induced association of GRB2 with tyrosine-phosphorylated 90-kD proteins, although some residual association of GRB2 with 90-kD proteins after activation of mutant Y764F was seen. This could be suggestive of an alternative less efficient mechanism of activation of 90-kD proteins. Notably, the 90-kD proteins were not found in Shc immunoprecipitates, suggesting that p90/GRB2 complexes are formed independently

of Shc and p145. The signaling molecule, Vav, with a molecular weight of 95 kD, has recently been shown to form complexes with GRB2 (53). We therefore investigated whether the 90-kD proteins could be Vav. However, reprobing of blots with anti-Vav antibodies indicated that p90 is distinct from Vav.

The phosphotyrosine phosphatase (PTP) SHP-2 (synonymous with Syp, PTP-1D, PTP-2C, SH-PTP2, or SH-PTP3) has been implicated in signal transduction mediated by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin, and the hematopoietic growth factors IL-3, GM-CSF, and IL-6 (24,25,49,54,55). SHP-2 binds to activated PDGF-R and EGF-R via SH2 domains and is then tyrosine-phosphorylated (24,25,56). Tyrosine phosphorylation of SHP-2 enhances its catalytic activity (25). The spectrum of activities of SHP-2 has not been fully elucidated. It has been suggested that SHP-2 acts as a negative regulator of receptor signal transduction by dephosphorylation of receptors, similar to the structurally related hematopoietic cell phosphatase (HCP) (57). However, in apparent contrast with this idea, SHP-2 does not efficiently dephosphorylate EGF-R or PDGF-R (25). Alternatively, SHP-2 could act as a positive regulator by dephosphorylating inhibitory phosphotyrosine sites. Recently, a novel function was attributed to SHP-2 when it was discovered that tyrosine-phosphorylated SHP-2 functions as an adapter between PDGF-R and GRB2 (36,38), resulting in recruitment of the GRB2/Sos complex to the PDGF-R/SHP-2 complex followed by activation of the $p21^{Ras}$ pathway (58). Our results suggest that SHP-2 may have a similar role in G-CSF-mediated signal transduction.

G-CSF-induced SHP-2/GRB2 association was severely reduced in cells expressing C-terminal deletion mutant d715 (containing only tyrosine 704) and was not detected in cells expressing deletion mutant d685 (lacking all cytoplasmic tyrosines). These findings established that the C-terminal region of G-CSF-R is required for association of GRB2 with activated SHP-2. However, in contrast to the formation of p145/Shc/GRB2 and p90/GRB2 complexes, G-CSF-induced SHP-2/GRB2 association did not predominantly depend on the presence of one particular tyrosine residue of G-CSF-R. This was evident from the observation that all tyrosine-to-phenylalanine substitution mutants of G-CSF-R were capable of inducing SHP-2/GRB2 complexes. Assuming that, in analogy with PDGF-R, SHP-2 acts as an adapter between G-CSF-R and GRB2, the association is partly induced via tyrosine 704 and partly via other tyrosine(s). Because GRB2 immunoprecipitates of cells expressing mutant Y764F contained tyrosine-phosphorylated SHP-2 but not the 90- and 145-kD proteins, the latter molecules are probably upstream of GRB2 in the signaling route, unless SHP-2 would efficiently dephosphorylate the 90- and/or 145-kD proteins or interfere with their binding to GRB2.

Collectively, our data could fit into a model in which recruitment of GRB2 to G-CSF-R occurs via multiple mechanisms, two of which depend on the presence of tyrosine 764 of G-CSF-R. Signaling via phosphotyrosine 764 of G-CSF-R is achieved through phosphorylation of either 145-kD or 90-kD proteins. In the first mechanism, phosphorylation of 145-kD proteins provides a binding site for the SH2 or PTB

domain of Shc. Shc is subsequently tyrosine-phosphorylated and becomes associated with GRB2. In the second mechanism, phosphorylated 90-kD proteins directly bind to GRB2 without the involvement of Shc. In the third mechanism, cytoplasmic phosphotyrosines of G-CSF-R bind SHP-2, which subsequently becomes tyrosine-phosphorylated, thereby providing a binding site for GRB2. Finally, a fourth possibility that still needs to be experimentally addressed is that GRB2 may bind directly to tyrosine residues of G-CSF-R.

The physiological significance of the different signaling mechanisms of G-CSF-R involving GRB2 remains to be established. It is anticipated that activation of the $p21^{Ras}$ pathway may occur via all these different mechanisms, involving multiple tyrosines of G-CSF-R. This would fit with the observation that a C-terminal truncation mutant of G-CSF-R, lacking tyrosine 764, is still capable of activation of $p21^{Ras}$ and MAP kinase (44). Activation of the $p21^{Ras}$/MAP kinase pathway by IL-3/GM-CSF receptors has recently been shown to be essential for supporting the survival of cells, by preventing apoptosis (59). It is likely that activation of this route through G-CSF-R serves a similar goal. Full understanding of the specific role of tyrosine 764 of G-CSF-R in signaling awaits elucidation of the identity and function of p90 and p145 proteins.

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CHAPTER 3

The membrane-distal cytoplasmic region of human granulocyte colony-stimulating factor receptor is required for STAT3 but not STAT1 homodimer formation

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Abstract

Signal transduction from the granulocyte colony-stimulating factor receptor (G-CSF-R) involves the activation of the Janus tyrosine kinase/signal transducer and activator of transcription (Jak/STAT) pathway. G-CSF induces tyrosine phosphorylation of Jak1, Jak2, STAT1, and STAT3. The membrane-proximal region of G-CSF-R is sufficient for activation of Jaks. It is still unclear how STAT proteins are activated by G-CSF-R. We investigated the possible involvement of the C-terminal region of G-CSF-R in the recruitment of STAT proteins using BAF3 cell transfectants expressing wild-type (WT) G-CSF-R, C-terminal deletion mutants and tyrosine-to-phenylalanine substitution mutants. Electrophoretic mobility shift assays with STAT-binding oligonucleotides (m67) showed that activation of WT G-CSF-R induces three distinct STAT complexes, namely STAT3 homodimers, STAT1-STAT3 heterodimers, and STAT1 homodimers. However, STAT1 homodimers and STAT1-STAT3 heterodimers were predominantly formed after activation of a C-terminal deletion mutant d685, which lacks all four conserved cytoplasmic tyrosine residues, located at positions 704, 729, 744, and 764. Antiphosphotyrosine immunoblots of STAT3 immunoprecipitates showed that activation of WT G-CSF-R induced phosphorylation of STAT3. In contrast, no phosphorylation of STAT3 was observed after activation of deletion mutant d685. These findings establish that the C-terminal region of G-CSF-R plays a major role in the activation of STAT3. By using tyrosine-to-phenylalanine substitution mutants of G-CSF-R, we further show that tyrosine 704, present in a YXXQ consensus sequence shown to be essential for STAT3 binding to gp130, is not exclusively involved in the activation of STAT3 by G-CSF-R.

Introduction

Granulocyte colony-stimulating factor (G-CSF) plays an essential role in the regulation of granulopoiesis and the maintenance of neutrophil levels in the peripheral blood. G-CSF stimulates the proliferation, survival, and maturation of cells committed to the neutrophilic granulocyte lineage (1,2). The diverse biological effects of G-CSF are mediated through a single class of cell-surface receptor proteins that form homodimeric complexes upon ligand binding. The human G-CSF receptor (G-CSF-R) is a member of the hematopoietin receptor superfamily (3,4). Although the intracellular domains of these receptors show little homology, certain similarities have been reported. For instance, the cytoplasmic membrane-proximal region of G-CSF-R, erythropoietin receptor (EPO-R), interleukin-6 (IL-6) signal transducer gp130, and the β chains of IL-2 and IL-3 receptors contains two conserved subdomains known as box 1 and box 2. This region is indispensable for transduction of growth signals (5-11). Importantly, the carboxy-terminal region of the cytoplasmic domain of G-CSF-R is involved in the induction of neutrophilic maturation (9,11).

The hematopoietin receptors lack intrinsic tyrosine kinase activity but activate cytoplasmic tyrosine kinases (2,4,12). Recently, signal transduction pathways that involve activation of Janus tyrosine kinases (Jaks) and signal transducer and activator of transcription (STAT) proteins have been linked to a number of receptor systems (13,14), including G-CSF-R (15-17). Jaks associate with the membrane-proximal cytoplasmic region of the hematopoietin receptors and become activated upon ligand-induced receptor homodimerization or heterodimerization (17-20). Jak activation leads to tyrosine phosphorylation of the STAT proteins. Activation of STATs involves phosphorylation of a conserved tyrosine residue just C-terminal of the Src homology 2 (SH2) domain (21). Subsequently, STAT proteins form stable homodimers and heterodimers by interactions between the SH2 domain of one STAT protein and the phosphotyrosine of another STAT protein before translocation to the nucleus, where they activate target genes by binding to specific regulatory sequences (22). How specific STAT proteins are recruited to the receptor/Jak complex in individual cytokine responses is still unclear.

G-CSF stimulation results in the rapid activation of STAT1 and STAT3, which leads to formation of STAT1 and STAT3 homodimeric and heterodimeric complexes (16). The C-terminal region of the human G-CSF-R contains 4 conserved tyrosine residues, located at positions 704, 729, 744, and 764, that form potential binding sites

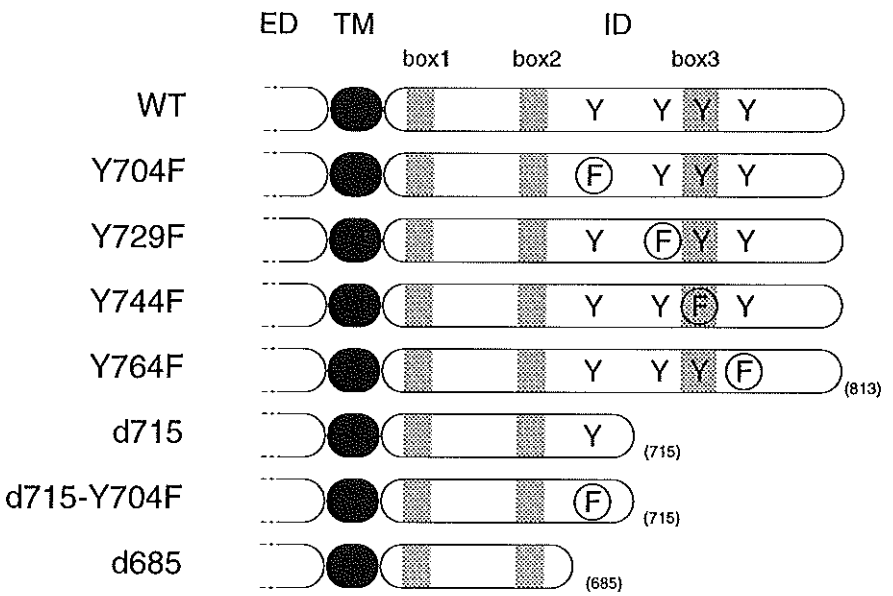

Figure 1. Schematic diagram of G-CSF-R proteins. Boxes 1, 2, and 3 denote subdomains conserved in some members of the hematopoietin receptor superfamily. Numbers in parentheses mark amino acid positions. ED, extracellular domain; TM, transmembrane domain; ID, intracellular domain; Y, tyrosine; F, phenylalanine.

for the SH2 domains of STATs. To establish the role of the C-terminal region of G-CSF-R in recruitment of STAT proteins, we studied STAT activation by wild-type (WT) G-CSF-R, C-terminal deletion mutants, and tyrosine-to-phenylalanine substitution mutants. We show that the C-terminal region of G-CSF-R is important for G-CSF-induced activation of STAT3 but not for STAT1 activation. Tyrosine 704, fitting the YXXQ consensus sequence for STAT3 binding to gp130 (23), is also involved in recruitment and activation of STAT3 by G-CSF-R. However, STAT3 activation is not exclusively accomplished via tyrosine 704; it is also accomplished via other domain(s) of the C-terminal region of G-CSF-R that do not fit the YXXQ consensus.

Materials and methods

G-CSF-R constructs and transfectants. Human *G-CSF-R* cDNA was cloned in the eukaryotic expression vector pLNCX (24). Polymerase chain reaction techniques were used to generate C-terminal deletion mutants d685 (M1) and d715 (DA); tyrosine-to-phenylalanine substitution mutants Y704F, Y729F, Y744F, and Y764F; and combined mutant d715-Y704F (Figure 1), as described previously (11,25). The IL-3-dependent murine pro-B cell line BAF3 (26) was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 ng/ml of murine IL-3. Transfection of BAF3 cells was performed by electroporation. G-CSF-R levels and G-CSF response of the BAF3 transfectants used have been described previously and were essentially similar to those of BAF3 cells expressing WT G-CSF-R (11,25).

Immunoprecipitation and Western blotting. Preparation of cell lysates, immunoprecipitation, and Western blotting were performed as described (25). Antibodies used for immunoprecipitation and Western blotting were anti-STAT3 antibodies (raised against amino acids 626-640; Santa Cruz Biotechnology Inc, Santa Cruz, CA), anti-STAT1α antibodies (raised against amino acids 716-739; Santa Cruz), and antiphosphotyrosine antibodies 4G10 (Upstate Biotechnology Inc, Lake Placid, NY).

Preparation of nuclear extracts. Before stimulation, cells were deprived of serum and factors for 4 hours at 37°C in RPMI 1640 medium. Cells (5 x 10^6/ml) were incubated in RPMI 1640 medium in the presence of human G-CSF (100 ng/ml) or murine IL-3 (1 μg/ml) for 5 to 60 minutes at 37°C or without factors (control). At the different time points, 10 vol of ice-cold phosphate-buffered saline with 1 mM Na_3VO_4 and 5 mM NaF 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 dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.2% Nonidet P-40, 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) (27). Cells were vortexed for 10 seconds, and nuclei were pelleted by centrifugation at 15,000*g* for 30 seconds. Nuclear extracts were prepared by resuspension of the nuclei in high-salt

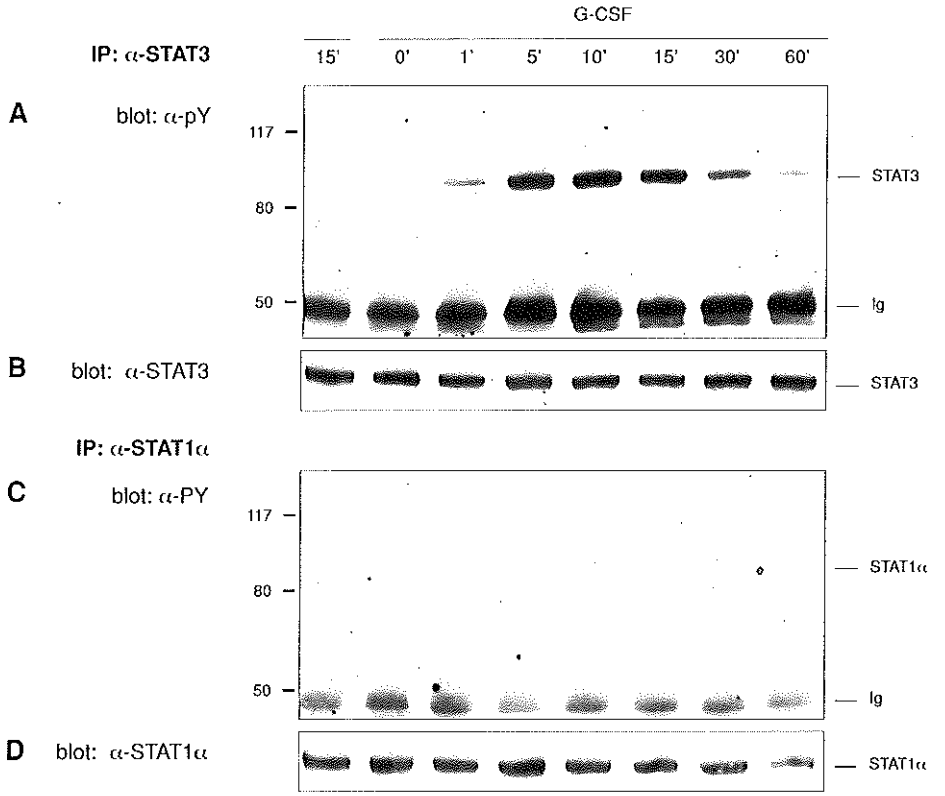

Figure 2. G-CSF-induced tyrosine phosphorylation of STAT3 and STAT1α in BAF3 cells expressing WT G-CSF-R. Serum- and growth factor-deprived cells were incubated at 37°C without factor (-) or with G-CSF (100 ng/ml) for the times indicated. STAT3 (A) or STAT1α (C) immunoprecipitates were subjected to Western blot analysis using antiphosphotyrosine antibodies 4G10. Blots were reprobed with anti-STAT3 (B) or anti-STAT1α (D) antibodies, respectively, to confirm equal loading. Positions of molecular mass standards (in kilodaltons [kD]) are indicated at the left. Ig, rabbit immunoglobulins.

buffer (hypotonic buffer with 420 mM NaCl and 20% glycerol) and extraction of proteins by rocking for 20 minutes at 4°C. Insoluble materials were removed by centrifugation for 20 minutes at 20,000*g* at 4°C.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts of 2 x 10^6 cells were incubated for 20 minutes at room temperature with 0.2 ng of ^{32}P-labeled double-stranded oligonucleotide (5 to 10,000 cpm) 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 dithiothreitol, 0.15 mM EDTA, 0.15 mM EGTA, 8% glycerol) (28). The oligonucleotide used in this study was m67 (5' CATTTCCCGTAAATC 3'), a high-

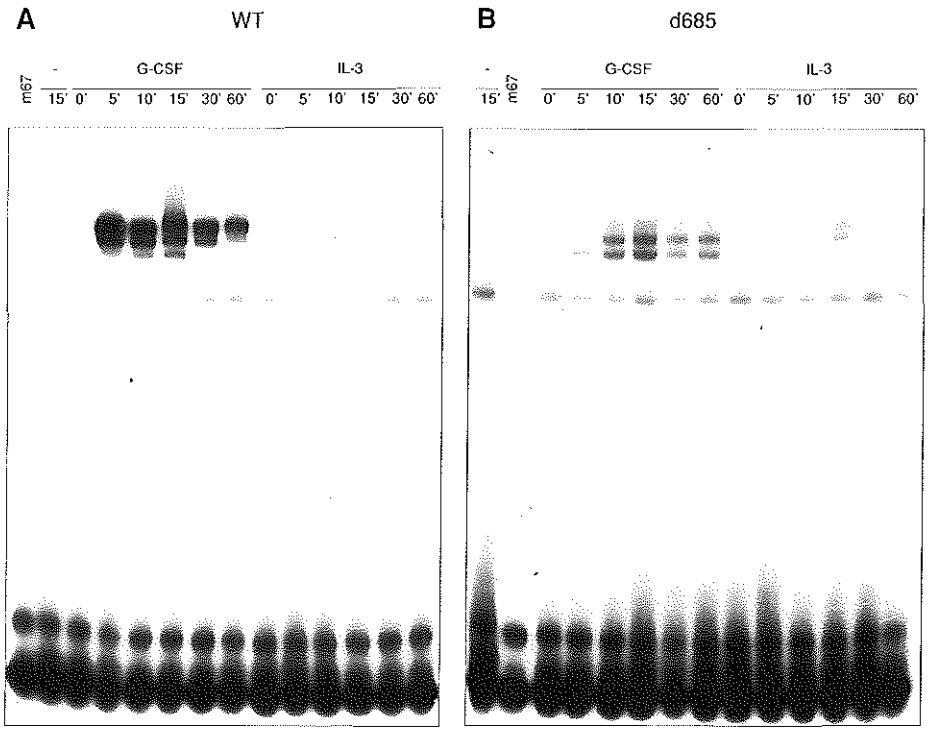

Figure 3. EMSA of nuclear extracts from BAF3 cells expressing WT G-CSF-R (A) or deletion mutant d685 (B). Serum- and growth factor-deprived cells were incubated at 37°C without factor (-), with G-CSF (100 ng/ml), or with IL-3 (1 µg/ml) for the times indicated. Nuclear extracts were prepared and incubated with ^{32}P-labeled double-stranded m67 oligonucleotide. m67, probe to which no nuclear extract was added.

affinity mutant of the sis-inducible element of the human *c-fos* gene (29). The DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gels containing 5% glycerol in 0.25x Tris-borate/EDTA electrophoresis buffer (TBE). The gels were dried and subsequently analyzed by autoradiography. For competition analysis, nuclear extracts were preincubated with 500-fold excess of unlabeled double-stranded m67 or Evi-1 oligonucleotide (30) in binding buffer for 20 minutes at room temperature before the addition of ^{32}P-labeled m67. For supershift analysis, nuclear extracts were preincubated for 2 hours on ice with 2 µg of anti-STAT3 (Santa Cruz), anti-STAT1α (Santa Cruz), or anti-STAT1αβ antibodies (raised against amino acids 688-710 mapping within the C-terminal sequence common to STAT1α and STAT1β; Santa Cruz) before the addition of ^{32}P-labeled m67 probe. Quantification of DNA-protein complexes was performed using the two dimensional Bio-Profil version 4.6 system (Vilber-Lourmat, France).

Results

Activation of WT G-CSF-R results in tyrosine phosphorylation of both STAT1 and STAT3 and in formation of three STAT1- and/or STAT3-containing DNA-binding complexes. To investigate whether STAT3 and STAT1α are tyrosine-phosphorylated after G-CSF treatment in BAF3 cells, STAT3 and STAT1α immunoprecipitates from unstimulated and G-CSF-stimulated BAF3 cells expressing WT G-CSF-R (BAF/WT) were probed with antiphosphotyrosine antibodies 4G10. As shown in Figure 2A, tyrosine phosphorylation of STAT3 was induced within 1 minute after G-CSF stimulation, then reached a maximum at 10 minutes, and persisted for at least 60 minutes. STAT1α was weakly and transiently phosphorylated after G-CSF treatment (Figure 2C). EMSAs with high-affinity sis-inducible element oligonucleotides (m67) showed that, after incubation of BAF/WT cells with G-CSF, formation of three distinct nuclear complexes was induced within 5 minutes (Figure 3A). Formation of the two slower migrating complexes was more prominent than that

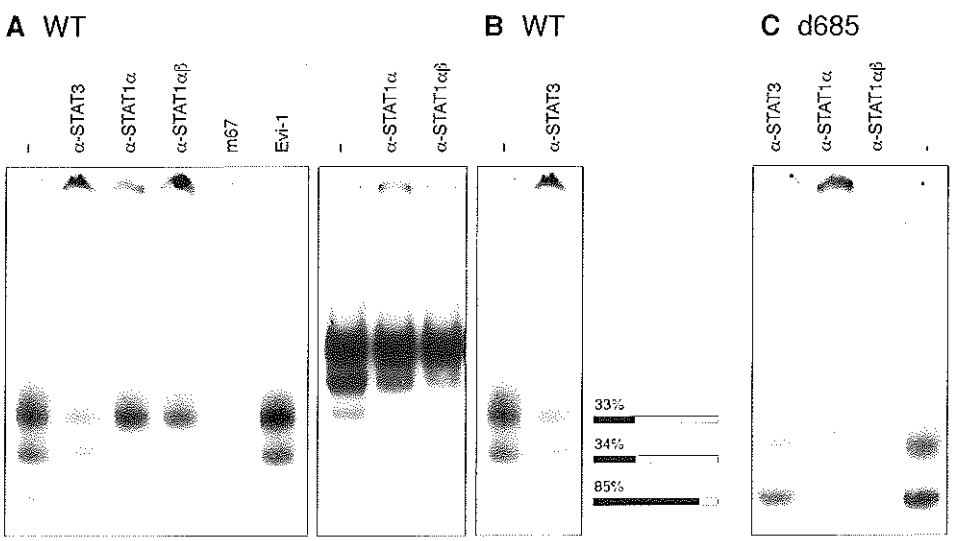

Figure 4. Supershift and competition analysis of G-CSF-induced DNA-binding complexes. Serum- and growth factor-deprived BAF3 cells expressing WT G-CSF-R (A and B) or deletion mutant d685 (C) were incubated for 15 minutes at 37°C with G-CSF (100 ng/ml). Nuclear extracts were prepared and preincubated without (-) or with antibodies or competitor oligonucleotides, as indicated, before the addition of ^{32}P-labeled m67 oligonucleotide. The intensity of the signals were quantified by densitometric analysis. Incubation with anti-STAT3 antibodies reduced the formation of the two slowest migrating complexes but did not affect the formation of the fastest migrating complex. Addition of anti-STAT1α or anti-STAT1αβ antibodies supershifted or blocked the formation of the two fastest migrating complexes but did not influence the formation and migration of the slowest migrating complex.

of the fastest migrating complex. Although not as intensely, IL-3 also induced formation of three DNA-binding complexes with apparently similar mobility as those of the G-CSF-induced complexes. Binding of the protein complexes to radioactive probe was effectively competed by an excess of unlabeled m67 oligonucleotide, but not by an excess of unrelated Evi-1 oligonucleotide, confirming the specificity of the DNA-protein interaction (Figure 4A). Incubation of nuclear extracts from BAF/WT cells with anti-STAT3 antibodies significantly reduced the formation of the two slower migrating complexes, whereas formation of the fastest migrating complex was marginally affected (Figure 4A). Inhibition of complex formation was calculated by expressing the amount of residual complex remaining after anti-STAT3 incubation as a percentage of the intensity of the complex to which no antibodies were added (Figure 4B). The two fastest migrating complexes supershifted with anti-STAT1α antibodies (Figure 4A). Moreover, formation of these complexes was completely abolished by anti-STAT1αβ antibodies. Neither anti-STAT1α nor anti-STAT1αβ antibodies affected the formation of the slowest migrating complex. These findings comply with results obtained with epidermal growth factor and IL-6-R systems and indicate that the slowest migrating complex consists of STAT3 homodimers, the complex with intermediate mobility of STAT1-STAT3 heterodimers and the fastest migrating complex of STAT1 homodimers (31,32).

The C-terminal region of G-CSF-R is involved in formation of STAT3 homodimers. To investigate a possible involvement of the C-terminal region of G-CSF-R in the formation of STAT complexes, nuclear extracts of BAF3 cells

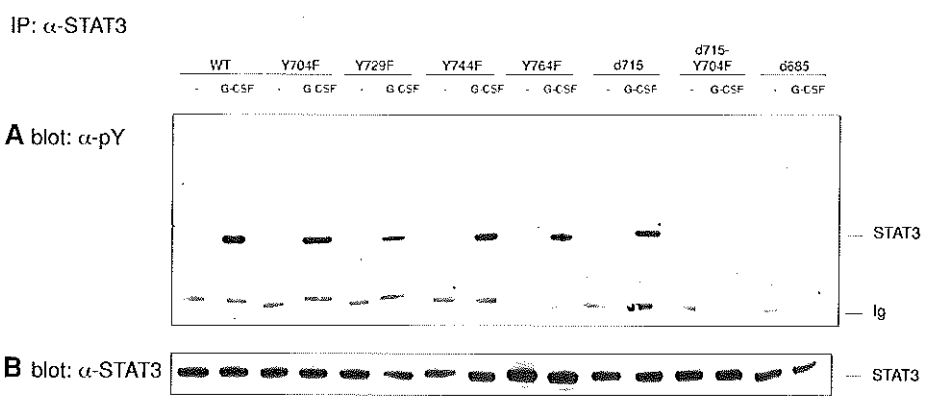

Figure 5. STAT3 immunoprecipitation on lysates from BAF3 cells expressing mutant G-CSF-R proteins. Serum- and growth factor-deprived cells were incubated for 15 minutes at 37°C without factor (-) or with G-CSF (100 ng/ml). (A) Western blot hybridized with antiphosphotyrosine antibodies 4G10. (B) Blot reprobed with anti-STAT3 antibodies to confirm equal loading of STAT3. Repeated analyses of at least three independent clones of each mutant gave identical results.

expressing C-terminal deletion mutant d685 (Figure 1) were analyzed. As expected, the mobility, amount, and kinetics of the IL-3-induced DNA-binding complexes were unaffected (Figure 3B). Whereas G-CSF primarily induced formation of STAT3 homodimers and STAT1-STAT3 heterodimers in BAF/WT cells, STAT1-STAT3 heterodimers and STAT1 homodimers were predominantly formed after activation of mutant d685, although some residual STAT3 homodimers were formed (Figures 3B and 4C). Furthermore, mutant d685 induced STAT3 homodimers significantly later in time as compared with that for WT G-CSF-R.

Tyrosine 704 and other domain(s) of the C-terminal region of G-CSF-R are involved in activation of STAT3. To determine which of the tyrosine residues located in the C-terminal region of G-CSF-R are involved in tyrosine phosphorylation of STAT3, BAF3 transfectants expressing C-terminal deletion or tyrosine substitution mutants of G-CSF-R (Figure 1) were studied in STAT3 immunoprecipitation assays. G-CSF induced tyrosine phosphorylation of STAT3 in cells expressing WT G-CSF-R or C-terminal deletion mutant d715, which contains tyrosine residue 704 (Figure 5). The observation that deletion mutant d715 is able to activate STAT3 is expected in view of the fact that tyrosine 704 is positioned within the STAT3-binding sequence YXXQ (23). In agreement with this, substitution of tyrosine 704 in mutant d715

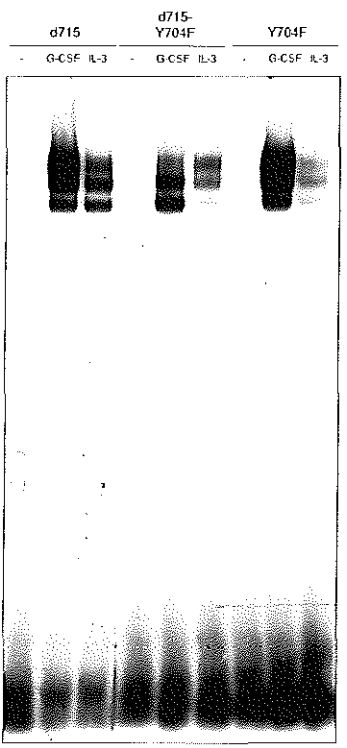

Figure 6. EMSA of nuclear extracts from BAF3 cells expressing mutant G-CSF-R proteins. Serum- and growth factor-deprived cells were incubated for 15 minutes at 37°C without factor (-), with G-CSF (100 ng/ml), or with IL-3 (1 μg/ml). Nuclear extracts were prepared and incubated with ^{32}P-labeled double-stranded m67 oligonucleotide.

(d715-Y704F) abolished its ability to activate STAT3. Substitution mutants Y704F, Y729F, Y744F, and Y764F all induced STAT3 activation, which indicates that tyrosine 704 is not exclusively involved in STAT3 recruitment to G-CSF-R (Figure 5). The (in)ability of the various deletion and substitution mutants to induce formation of STAT3 homodimers as determined by EMSA was in agreement with the STAT3 immunoprecipitation results. G-CSF induced STAT3 homodimer formation in cells expressing C-terminal deletion mutant d715 and substitution mutant Y704F, whereas formation of STAT3 homodimers was severely reduced after activation of combined mutant d715-Y704F (Figure 6). As expected, activation of substitution mutants Y729F, Y744F, and Y764F resulted in STAT3 homodimer formation (data not shown).

Discussion

The Jak/STAT signal transduction pathway is activated by a number of hematopoietin receptors. The membrane-proximal cytoplasmic regions of G-CSF-R, EPO-R, and other members of the hematopoietin receptor family have been shown to be primarily responsible for the activation of Jaks (17-19). For some receptors, e.g. EPO-R and the β_c chain of IL-3-R and GM-CSF-R, it was shown that Jak proteins physically associate with the membrane-proximal region (18,20). In EPO-R, a single conserved tryptophan residue in this region is crucial for binding of Jak2 (33). A central question concerns the mechanisms of recruitment of STATs to the receptor/Jak complexes. It has been proposed that Jaks differentially phosphorylate STATs via the involvement of the cytoplasmic domains of the receptor proteins in recruitment of specific STAT proteins. For instance, in the cytoplasmic domain of the interferon-γ receptor α chain tyrosine residue 440 is required for STAT1 phosphorylation and activation, presumably through specific interaction with the SH2 domain of STAT1 (34,35). Similarly, STAT3 is activated via interaction with multiple tyrosine residues in the cytoplasmic domain of gp130 and leukemia inhibitory factor receptor (23), and IL-4-induced activation of STAT6 is mediated by tyrosines 578 and 606 of IL-4-R (36).

In this study, we established that the membrane-distal cytoplasmic region of G-CSF-R containing four conserved tyrosine residues is involved in the activation of STAT3. In contrast, activation of STAT1 is independent on this region of G-CSF-R and, thus, is not determined by phosphotyrosine residues of the receptor. Similarly, activation of STAT1 (or a related molecule) by growth hormone (37) and of STAT5 by GM-CSF (38) does not require tyrosine phosphorylation of the receptor. It has been proposed that Jak1 and Jak2 specifically recruit and phosphorylate STAT1 and STAT5, respectively (39,40). In these cases, it is possible that STATs associate with tyrosine-phosphorylated Jaks through their SH2 domains. Alternatively, additional STAT domains could be involved in recruitment of STATs to the receptor/Jak complex.

Formation of STAT1-STAT3 heterodimeric and of STAT1 homodimeric complexes was unaffected by deletion of the membrane-distal cytoplasmic region of G-CSF-R, whereas formation of STAT3 homodimers was severely reduced. Recently, it was shown that interferon-α stimulation induces the formation of STAT1-STAT3 heterodimeric and STAT1 homodimeric complexes without STAT3 homodimer formation (41,42). These data could fit into a model in which formation of stable STAT1-STAT3 heterodimeric complexes occurs via sequential activation in analogy with the proposed mechanism of ISGF3 formation after interferon-α stimulation (43). STAT1 is recruited to the receptor/Jak complex and is subsequently phosphorylated, thereby providing a binding site for the SH2 domain of STAT3. After association of STAT3 with STAT1, STAT3 is phosphorylated, followed by stable STAT1-STAT3 heterodimer formation and release from the receptor/Jak complex. If a second STAT3 protein associates with the first tyrosine-phosphorylated STAT3 protein before release of the STAT1-STAT3 heterodimer, some residual STAT3 homodimeric complexes could be formed, as we indeed observed (Figure 3B). Although STAT1-STAT3 heterodimeric complexes were formed after activation of the C-terminal deletion mutant d685, tyrosine phosphorylation of STAT3 was not detected in antiphosphotyrosine immunoblots of STAT3 immunoprecipitates. This most likely reflects the lower sensitivity of the immunoprecipitation techniques as compared with that of EMSA.

To investigate whether the conserved cytoplasmic tyrosine residues are involved in the activation of STAT3 by the C-terminal region of G-CSF-R, tyrosine-to-phenylalanine substitution mutants were studied. Tyrosine 704 of G-CSF-R fits the YXXQ consensus sequence for STAT3 binding (23) and, indeed, appeared to be involved in recruitment of STAT3. STAT3 was initially characterized as the acute-phase response factor, because of its association with the IL-6-induced expression of acute-phase genes (44-46). Identification of tyrosine 704 of G-CSF-R as a STAT3 activation site provides an explanation for the results that a region surrounding tyrosine 704 (amino acids 686-725) is capable of inducing acute-phase plasma protein gene expression when G-CSF-R is expressed in human hepatoma cell lines (10). It is not known whether the SH2 domain of STAT3 directly binds to the YXXQ sequence in receptor proteins or whether STAT3 associates with the receptor via an adapter protein that interacts with the YXXQ sequence. Although tyrosine 704 is the only cytoplasmic tyrosine of G-CSF-R fitting the YXXQ consensus sequence, STAT3 activation was not exclusively mediated via tyrosine 704. This was evident from the observation that all tyrosine-to-phenylalanine substitution mutants of full-length G-CSF-R were capable of inducing STAT3 phosphorylation. Assuming that STAT3 recruitment is regulated by phosphotyrosine-SH2 domain interactions, at least one of the other cytoplasmic tyrosine residues of G-CSF-R not fitting the YXXQ consensus sequence provides a direct or indirect binding site for STAT3. The YXXQ sequence differs from the sequences in STAT3 (YLKT) and STAT1 (YIKT) that are implicated in mediating homodimerization or heterodimerization (22,45,46). Thus, YXXQ is not the only consensus sequence to which the SH2 domain of STAT3 can bind,

suggesting possible involvement of other tyrosines of G-CSF-R in direct binding to STAT3. Alternatively, phosphotyrosine(s) or other domain(s) of the C-terminal region of G-CSF-R may interact with an adapter protein containing a YXXQ sequence that is phosphorylated after G-CSF stimulation, thereby providing a binding site for the SH2 domain of STAT3.

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CHAPTER 4

Tryptophan 650 of human granulocyte colony-stimulating factor (G-CSF) receptor, implicated in the activation of Jak2, is also required for G-CSF-mediated activation of signaling complexes of the $p21^{Ras}$ route

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Abstract

Granulocyte colony-stimulating factor (G-CSF) induces rapid phosphorylation of Jak kinases as well as activation of the $p21^{Ras}$ route through interaction with its specific receptor (G-CSF-R). The cytoplasmic membrane-proximal region of G-CSF-R (amino acids 631 to 685) is necessary for proliferation induction and activation of Jak2. In contrast, activation of Shc and SHP-2, signaling molecules implicated in the $p21^{Ras}$ signaling route, depends on the phosphorylation of tyrosine residues located in the membrane-distal region (amino acids 686 to 813) of G-CSF-R. We investigated whether G-CSF-induced activation of signaling complexes of the $p21^{Ras}$ route depends on the function of the membrane-proximal cytoplasmic region of G-CSF-R. A G-CSF-R mutant was constructed in which tryptophan 650 was replaced by arginine and expressed in BAF3 cells (BAF/W650R). In contrast to BAF3 cell transfectants expressing wild-type G-CSF-R, BAF/W650R cells did not proliferate and did not show activation of Jak2, STAT1, or STAT3 in response to G-CSF. Immunoprecipitations with anti-Shc and anti-GRB2 antisera showed that mutant W650R also failed to activate SHP-2 and Shc. These data indicate that the membrane-proximal cytoplasmic domain of G-CSF-R is not only crucial for proliferative signaling and activation of Jak2 and STATs, but is also required for activation of the $p21^{Ras}$ route, which occurs via the membrane-distal region of G-CSF-R.

Introduction

Granulocyte colony-stimulating factor (G-CSF) regulates the proliferation, differentiation, and survival of myeloid progenitor cells (1). The diverse biological effects of G-CSF are mediated through binding of G-CSF to a specific receptor (G-CSF-R) that belongs to the hematopoietin receptor superfamily (2,3). The cytoplasmic domains of these receptors have no intrinsic kinase function and show very little overall sequence homology. The membrane-proximal cytoplasmic region of several members of this receptor superfamily, including G-CSF-R, contains two distinct subdomains designated as box 1 and box 2. This region is indispensable for transduction of mitogenic signals (4-6).

For most cytokine receptors it has now been shown that they activate cytoplasmic tyrosine kinases of the Janus kinase (Jak) family. Activation of Jak kinases is mediated via the membrane-proximal cytoplasmic region of the receptor. Subsequently, Jaks phosphorylate STAT (signal transducer and activator of transcription) proteins, which form homodimers and/or heterodimers, translocate to the nucleus, and activate target genes by interaction with specific DNA sequences (7-12). For erythropoietin receptor (EPO-R), interleukin-6 (IL-6) and IL-11 signal transducer gp130, and the common β chain of granulocyte-macrophage colony-stimulating factor receptor (GM-CSF-R), IL-3-R, and IL-5-R (β_c), it has been shown that the cytoplasmic membrane-proximal region of the receptor physically interacts

with the Jak proteins (13-15). Mutational analysis of EPO-R showed that a tryptophan residue (W282) located between box 1 and box 2 is essential for Jak2 activation and proliferative signaling of EPO-R (16). This tryptophan (Trp) is conserved in several proteins of the cytokine receptor superfamily, e.g. IL-2-R β chain, IL-4-R, G-CSF-R, gp130, and β_c. The essential role of Jak2 activation in mitogenesis was recently established using a kinase-deficient form of Jak2 in an EPO-dependent cell line (17).

The cytoplasmic domain of G-CSF-R contains four conserved tyrosines, all located in the membrane-distal region. Upon phosphorylation, these tyrosine residues form potential binding sites for signaling molecules that contain SH2 domains. Previously, it has been shown that, like IL-3 and EPO, G-CSF activates $p21^{Ras}$, MAP kinase, and a variety of SH2-containing signaling intermediates of the $p21^{Ras}$/MAP kinase route, e.g. Shc and the phosphotyrosine phosphatase SHP-2 (Syp) (18-21). This occurs via the membrane-distal cytoplasmic region of G-CSF-R (18). It has not been established whether phosphorylation of these substrates depends on the integrity of box1/box2 region, implicated in the activation of Jak2 (10).

In the present study, we have investigated the functional consequences of a single amino acid substitution (W650R), changing a Trp to an arginine (Arg) in the membrane-proximal cytoplasmic region of G-CSF-R. We show that this mutation completely abolishes the mitogenic response to G-CSF. No Jak2 phosphorylation and subsequent formation of STAT complexes could be detected on activation of the W650R mutant. Significantly, the ability of G-CSF-R to induce formation of p145/Shc/GRB2, p90/GRB2, and SHP-2/GRB2 complexes is also completely abrogated by the W650R substitution. Thus, we show that the membrane-proximal cytoplasmic region of G-CSF-R is not only crucial for G-CSF-R-mediated signaling via the Jak/STAT pathway, but also for activation of signaling complexes of the $p21^{Ras}$ route, which occurs via the carboxy-terminal region of G-CSF-R.

Materials and methods

G-CSF-R expression constructs. Human *G-CSF-R* cDNA was cloned in the pBluescript (pBS) vector and in the eukaryotic expression vector pLNCX (6). G-CSF-R mutation W650R was obtained by polymerase chain reaction (PCR)-mediated site-directed mutagenesis, as described previously (18). Human *G-CSF-R* cDNA cloned in pBS was used in the first PCR reaction as the template with the oligonucleotide 5' GGGCTCCCGGGTACCCACAATCAT 3' as the mutagenic primer (altered bases are underlined) together with the primer M13-20 (5' GTAAAACGACGGCCAGT 3'), a primer specific for pBS. In the second PCR, human *G-CSF-R* cDNA cloned in pLNCX was used as the template with the product of the first PCR as a megaprimer and with primers M13-20 and FR2 (5' TGTGATCATCGTGACTCCCTT 3'). The PCR product was gel-purified and inserted into pLNCX. DNA sequencing was performed to confirm the presence of the introduced mutation and the integrity of the insert.

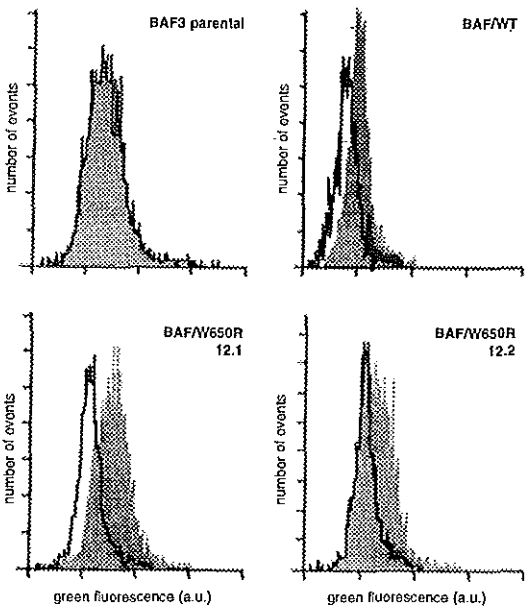

Figure 1. Flow cytometric analysis of G-CSF-R expression on parental BAF3 cells and BAF3 transfectants. Cells were stained with either rabbit anti-G-CSF-R antibodies and GAR/FITC (grey area) or with GAR/FITC alone (open area).

Cells and DNA transfection. The IL-3-dependent murine pro-B cell line BAF3 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and murine IL-3 (10 ng/ml) (22). The pLNCX expression constructs were linearized by *Pvu*I digestion and transfected into BAF3 cells by electroporation. After gene transfer, neomycin-resistant clones were selected in IL-3-containing medium supplemented with 1.5 mg/ml G418 (GIBCO-BRL, Breda, The Netherlands) and expanded for further analysis. To determine G-CSF-R expression levels, cells were treated with rabbit antiserum against the extracellular domain of human G-CSF-R and fluorescein isothiocyanate-labeled goat antirabbit IgG (GAR/FITC; Nordic Immunology, Tilburg, The Netherlands) and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). To estimate the affinities of the G-CSF binding sites, ^{125}I-G-CSF binding experiments and Scatchard analysis were performed as described (6).

DNA synthesis assay. DNA synthesis was assessed by [^{3}H]-thymidine (^{3}H-TdR) uptake. Cells (10^4) were incubated in triplicate in 100 µl of 10% FCS-RPMI medium supplemented with titrated concentrations of human G-CSF or with 10 ng/ml murine IL-3 in 96-well plates for 48 hours. Sixteen hours before cell harvest, 0.1 µCi of ^{3}H-TdR (2 Ci/mM; Amersham International, Amersham, UK) was added to each well. ^{3}H-TdR incorporation was measured by liquid scintillation counting.

Immunoprecipitation and Western blotting. Cells (2 x 10^7) were deprived of serum and growth factors for 4 hours at 37°C. One hour before the addition of growth factors, 10 μM Na_3VO_4 was added. Cells were incubated for 10 minutes at 37°C in the presence of G-CSF (100 ng/ml) or IL-3 (1 μg/ml) or without factor. The reaction was terminated with ice-cold phosphate-buffered saline. Cells were lysed by incubation for 30 minutes at 4°C in lysis buffer. Insoluble materials were removed by centrifugation for 15 minutes at 10,000*g* at 4°C. For Jak2 immunoprecipitations, a lysis buffer was used that contained 50 mM Tris [pH 8.0], 50 mM NaF, 200 mM NaCl, 10% glycerol, 0.5% (vol/vol) Triton X-100, 0.1 mM Na_3VO_4, and 1 mM Pefabloc SC, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml bacitracin, and 50 μg/ml iodoacetamide as protease inhibitors. For Shc and GRB2 immunoprecipitations, we used a lysis buffer containing 20 mM Tris [pH 8.0], 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% Nonidet P-40, 1 mM DTT, 10% glycerol, 2 mM Na_3VO_4, and the cocktail of protease inhibitors. Cell lysates were incubated overnight at 4°C with rabbit polyclonal anti-Jak2 antiserum (kindly provided by Dr. J.N. Ihle, Memphis, TN), anti-GRB2 (Santa Cruz Biotechnology Inc, Santa Cruz, CA), or anti-Shc (kindly provided by Dr. J.L. Bos, Utrecht, The Netherlands) antibodies. Protein A-Sepharose beads (Pharmacia LKB, Uppsala, Sweden) were added for 1 hour at 4°C. After five washings in lysis buffer, proteins were eluted from the beads by boiling in sodium dodecyl sulfate (SDS)-sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and electrotransferred onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Filters were blocked by incubation in 0.3% Tween-20 in Tris-buffered saline (TBS; 10 mM Tris [pH 7.4], 150 mM NaCl) for 1 hour at 37°C, washed in TBS containing 0.05% Tween-20 (TBS-T), and incubated with various antibodies. The antibodies used for Western blotting were antiphosphotyrosine antibody 4G10 (Upstate Biotechnology Inc, Lake Placid, NY), anti-Shc, anti-GRB2, anti-Jak2, and anti-SHP-2 (Santa Cruz). After five washings in TBS-T, the filters were probed with horseradish peroxidase-conjugated species specific antiserum (DAKO A/S, Glostrup, Denmark), followed by enhanced chemiluminescence reaction (DuPont, Boston, MA). For

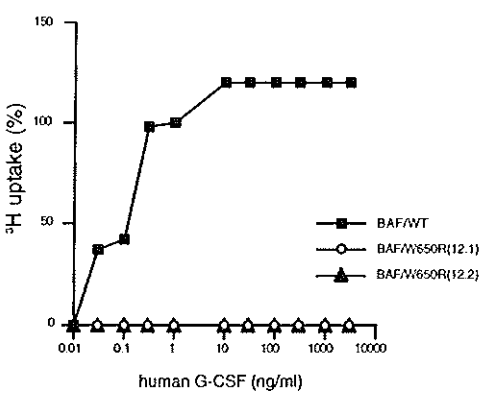

Figure 2. G-CSF responsiveness of BAF/WT and BAF/W650R cells in ^{3}H-TdR uptake assays. For each clone, data are expressed as the percentage of ^{3}H-TdR incorporation in cells stimulated with 10 ng/ml of murine IL-3 (approximately 15,000 ± 500 cpm for all clones tested).

reprobing with different antibodies, blots were stripped in 62.5 mM Tris-HCl [pH 6.7], 2% SDS, and 100 mM β-mercaptoethanol at 50°C for 30 minutes and reblocked with 0.3% Tween-20 in TBS.

Gel retardation assays. Nuclei of 2 x 10^6 cells were obtained by lysis in a 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% Nonidet P-40, 0.125 μM okadaic acid, and the cocktail of protease inhibitors). After lysis of the cells, nuclei were precipitated by centrifugation at 15,000*g* for 30 seconds. Nuclear extracts were prepared using a high-salt buffer (hypotonic buffer with 420 mM NaCl and 20% glycerol). Insoluble materials were removed by centrifugation for 20 minutes at 20,000*g* at 4°C. The extracts were incubated at room temperature for 20 minutes with 0.2 ng of ^{32}P-labeled double-stranded STAT-binding oligonucleotide and 2 μg of poly(dI-dC) in 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, 8% glycerol) (23). The oligonucleotide used was m67 (5' CATTTCCCGTAAATC 3') (24). DNA-protein complexes were separated on 5% nondenaturing polyacrylamide gels in 0.25x TBE and visualized by autoradiography.

Results

Expression of wild-type and mutant G-CSF-R in BAF3 transfectants. Expression of wild-type (WT) and mutant (W650R) G-CSF-R proteins in BAF3 transfectants was first examined by FACScan analysis using G-CSF-R antibodies (Figure 1). Two representative BAF/W650R clones (12.1 and 12.2) and one BAF/WT clone are shown. Parental BAF3 cells did not bind G-CSF-R antibodies. Binding studies with ^{125}I-G-CSF indicated that the BAF/WT cells express a mean number of 2,750 sites per cell with a K_d of 3.3 nM. BAF/W650R clone 12.2 cells expressed 1,750 sites per cell with a K_d of 2.2 nM.

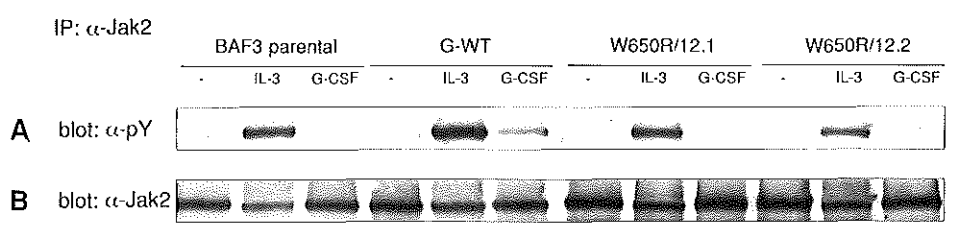

Figure 3. Jak2 immunoprecipitates of parental BAF3 cells and BAF3 transfectants. Growth factor-deprived cells were incubated for 10 minutes at 37°C without factor (-), with G-CSF (100 ng/ml), or with IL-3 (1 μg/ml). (A) Western blot hybridized with antiphosphotyrosine antibodies. (B) Blot reprobed with anti-Jak2 antibodies to confirm equal loading of Jak2.

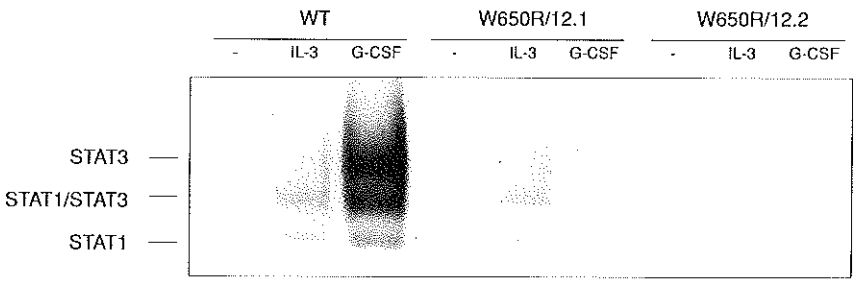

Figure 4. Gel retardation assay with nuclear extracts from BAF/WT and BAF/W650R cells. Serum- and growth factor-deprived cells were incubated for 15 minutes at 37°C without factor (-), with G-CSF (1 µg/ml), or with IL-3 (1 µg/ml). Nuclear extracts were prepared and incubated with ^{32}P-labeled m67 probe. The three nuclear STAT complexes are indicated.

G-CSF fails to induce proliferation of BAF/W650R transfectants. Expression of WT G-CSF-R in BAF3 cells confers G-CSF-dependent proliferation. The ability of G-CSF to induce proliferative signals in BAF/W650R cells was examined by ^{3}H-TdR uptake assays (Figure 2). BAF/WT clones responded to G-CSF in a dose-dependent manner, with a maximal response at 3 ng/ml G-CSF. In contrast, BAF/W650R transfectants did not respond to G-CSF, even at high concentrations of G-CSF (3.3 µg/ml). Four independent W650R clones gave identical results. In contrast to BAF/WT cells that proliferate and can be maintained in the presence of G-CSF as the single growth factor, BAF/W650R cells died within 24 hours in G-CSF-supplemented medium (data not shown).

G-CSF fails to activate Jak2 and STATs in BAF/W650R cells. G-CSF induced tyrosine phosphorylation of Jak2 in BAF/WT cells (Figure 3). In contrast, G-CSF failed to activate Jak2 in BAF/W650R transfectants. In parallel control incubations, IL-3 induced tyrosine phosphorylation of Jak2 in both BAF/WT and BAF/W650R transfectants. Gel retardation assays with STAT binding m67 oligonucleotides showed that three STAT complexes are formed in BAF/WT cells treated with G-CSF (Figure 4). These three complexes consist of STAT1 homodimers, STAT1/STAT3 heterodimers, and STAT3 homodimers (12). In contrast, G-CSF failed to induce STAT1 and/or STAT3 DNA binding in BAF/W650R cells, whereas IL-3 did.

G-CSF also fails to activate Shc-, GRB2-, and SHP-2-containing complexes in BAF/W650R cells. Shc immunoprecipitates from G-CSF-stimulated BAF/WT cells contained tyrosine-phosphorylated Shc and p145 (Figure 5). In contrast, no activation of Shc and p145 was seen after G-CSF stimulation in BAF/W650R transfectants, despite the equal presence of Shc proteins as compared with BAF/WT cells. IL-3 induced phosphorylation of Shc and p145 in all of these transfectants. After incubation of BAF/WT cells with G-CSF and IL-3, tyrosine-phosphorylated Shc, SHP-2, p90, and p145 coprecipitated with GRB2 (Figure 6). G-CSF stimulation of BAF/W650R transfectants did not induce association of GRB2 with phosphorylated

Shc, SHP-2, p90, or p145, whereas in parallel control incubations, IL-3 did induce the formation of these complexes (Figure 6).

Discussion

In previous investigations, distinct functional regions in the cytoplasmic domain of G-CSF-R have been identified (5,6,25). It was found that the membrane-distal region of approximately 100 amino acids is required for the induction of neutrophilic maturation, whereas a membrane-proximal region of 55 amino acids is essential for mitogenic signaling. This latter region contains the box 1 and 2 sequences conserved among several members of the hematopoietin receptor superfamily. Site-directed mutagenesis of G-CSF-R confirmed the importance of box 1 in proliferative signaling of G-CSF. Replacement of Pro-638 and Pro-640 of human G-CSF-R and replacement of Trp-634, Pro-638, and Pro-640 and Asp-639 located within box 1 of murine G-CSF-R completely inactivated G-CSF-induced mitogenesis (5,26). The mechanism by which these substitutions result in the inactivation of the receptor is not known.

The present study was initiated to determine the importance of Trp-650 of G-CSF-R, positioned between box 1 and box 2, for signal transduction. We have shown that substitution of Arg for Trp at this position completely abolishes the mitogenic signaling abilities of G-CSF-R as well as its capacity to induce Jak2 phosphorylation. Binding studies with ^{125}I-G-CSF showed that mutant W650R had a similar affinity for the ligand, indicating that the loss of G-CSF responsiveness cannot be explained by a loss of G-CSF binding to mutant W650R-receptor.

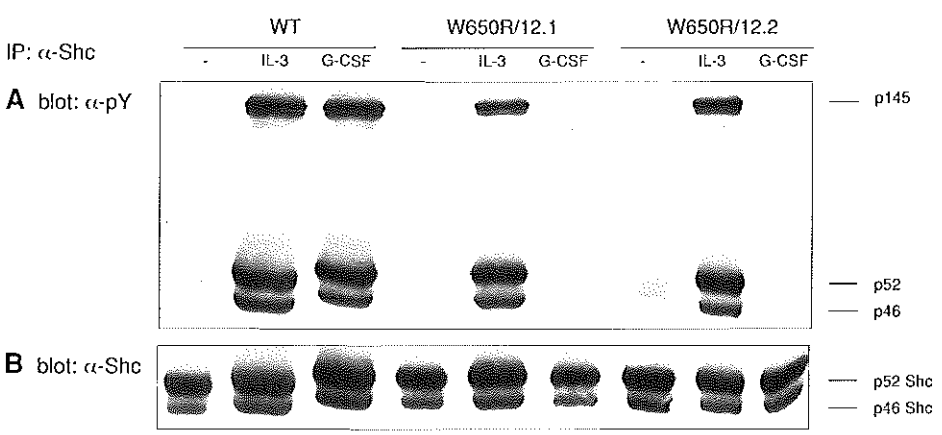

Figure 5. Shc immunoprecipitates of BAF/WT and BAF/W650R cells. Serum- and growth factor-deprived cells were incubated for 10 minutes at 37°C without factor (-), with G-CSF (100 ng/ml), or with IL-3 (1 µg/ml). (A) Western blot hybridized with antiphosphotyrosine antibodies. (B) Blot reprobed with anti-Shc antiserum to confirm equal loading of Shc.

Studies on the EPO-R have shown that Jak2 physically associates with the membrane-proximal domain of the receptor (13). In analogy with the observations in EPO-R, in which a similar amino acid substitution (W282R) completely inactivates proliferative signaling by EPO-R and disrupts the interaction between Jak2 and EPO-R (16), we assume that mutant W650R fails to bind Jak2. The exact mechanism of Jak association with G-CSF-R is not yet clear. Jak kinases do not contain SH2 or SH3 domains, motifs that are essential for the interaction of a variety of cytoplasmic protein tyrosine kinases with receptor molecules. In EPO-R, box 1 as well as the region between box 1 and 2 are required for binding of Jak2 (13). Similarly, Jak2 binding to the common β chain of GM-CSF-R, IL-3-R, and IL-5-R requires the box 1 region and the downstream 14 amino acids, which include the conserved Trp residue (15). In contrast, in prolactin receptor, also a member of the cytokine receptor superfamily, box 1 is sufficient for Jak2 activation. In this receptor, a deletion immediately C-terminal of box 1 has no effect on the interaction with Jak2 (27). Notably, prolactin receptor does not contain the conserved Trp. Therefore, it is possible that association of Jak2 with different receptors involves distinct amino acid residues in addition to box 1.

Recent findings in our laboratory have shown that a G-CSF-R mutant d685 (M1), that lacks all four cytoplasmic tyrosine residues, is unable to activate Shc and

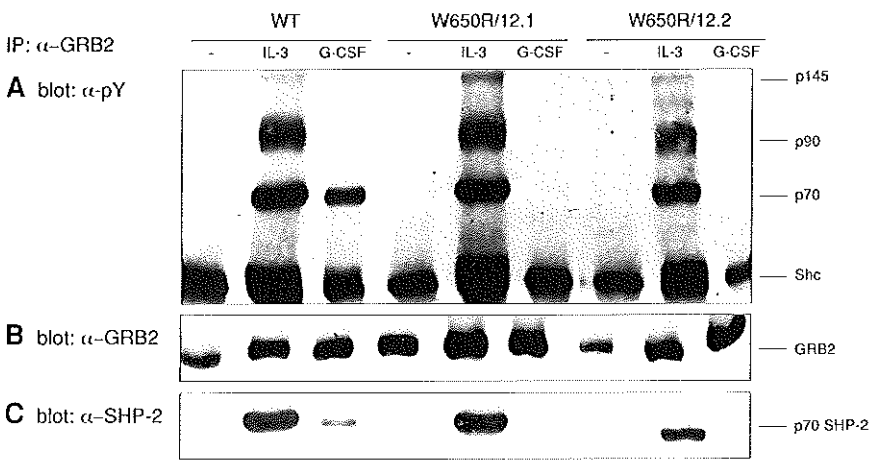

Figure 6. GRB2 immunoprecipitates of BAF/WT and BAF/W650R cells. Serum- and growth factor-deprived cells were incubated for 10 minutes at 37°C without factor (-), with G-CSF (100 ng/ml), or with IL-3 (1 μg/ml). (A) Western blot hybridized with antiphosphotyrosine antiserum. The band at the bottom is predominantly heavy chain of the anti-GRB2 antibodies that clouds the Shc signal in this exposure. In a shorter exposure, tyrosine-phosphorylated Shc is visible on top of this band in all clones stimulated with IL-3 and in BAF/WT cells stimulated with G-CSF. (B) Western blot hybridized with anti-GRB2 antiserum to confirm equal loading of GRB2. (C) Blot reprobed with anti-SHP-2 antiserum.

SHP-2 (18). Many growth factors induce phosphorylation of Shc, sequentially resulting in formation of the Shc/GRB2/Sos complex, $p21^{Ras}$-GTP formation, and cellular responses. It has been shown that tyrosine 764 of G-CSF-R is essential for G-CSF-induced Shc phosphorylation and its association with p145 and GRB2 (18). SHP-2, like Shc, can link GRB2 to receptor proteins, thereby providing a mechanism for activation of $p21^{Ras}$. For instance, SHP-2 couples GRB2 to phosphorylated tyrosine 1009 of PDGF-R (28), a tyrosine involved in PDGF-induced activation of the $p21^{Ras}$ route (29).

Results from the present study and our previous findings (18) fit into the following model. Upon G-CSF binding, wild-type G-CSF-R activates Jak2, which subsequently phosphorylates the tyrosine residues within the C-terminal region of the receptor. These phosphotyrosines then form binding sites for the SH2-containing signaling molecules involved in the $p21^{Ras}$ route, such as Shc and SHP-2. Mutant W650R fails to bind and activate Jak2. Consequently, the cytoplasmic tyrosines of G-CSF-R will not be phosphorylated and thereby cannot recruit p145/Shc/GRB2, p90/GRB2, or SHP-2/GRB2 complexes to the receptor.

Although this and other studies (16,17) strongly suggest that Jak kinases are crucial for proliferative signaling of hematopoietin receptors and that activation of Jaks is required for activation of $p21^{Ras}$ route, it is still not completely clear to what extent the Jak/STAT and $p21^{Ras}$ signaling pathways converge and contribute to the distinct cellular responses induced via G-CSF-R. Recently, expression of a dominant-inhibitory mutant of c-Ha-ras in 32Dcl3 cells was found to interfere with IL-3-induced proliferation (30). In contrast, G-CSF could still induce neutrophilic maturation in these cells, suggesting that activation of the $p21^{Ras}$ pathway per se is not required for maturation induction (30). Extension of these investigations, for instance with the use of G-CSF-responsive 32D cell transfectants in which both Jak and $p21^{Ras}$ function can be sequentially or simultaneously altered by dominant-negative mutants, may help to further elucidate the complex interplay between Jak/STAT and $p21^{Ras}$ signaling pathways in G-CSF-R-mediated proliferation, maturation, and survival of myeloid precursor cells.

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CHAPTER 5

Proliferation signaling and activation of Shc, $p21^{Ras}$, and Myc via tyrosine 764 of human granulocyte colony-stimulating factor receptor

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Abstract

The membrane-distal region of the cytoplasmic domain of human granulocyte colony-stimulating factor receptor (G-CSF-R) contains four conserved tyrosine residues: Y704, Y729, Y744, and Y764. Three of these (Y729, Y744, and Y764) are located in the C-terminal part of G-CSF-R, previously shown to be essential for induction of neutrophilic differentiation. To determine the role of the tyrosines in G-CSF-mediated responses, we constructed tyrosine-to-phenylalanine (Y-to-F) substitution mutants and expressed these in a differentiation competent subclone of 32D cells that lacks endogenous G-CSF-R. We show that all tyrosines can be substituted essentially without affecting the differentiation signaling properties of G-CSF-R. However, substitution of one specific tyrosine, i.e. Y764, markedly influenced proliferation signaling as well as the timing of differentiation. 32D cells expressing wild-type (WT) G-CSF-R (or mutants Y704F, Y729F, or Y744F) proliferated in G-CSF-containing cultures until day 8 and then developed into mature neutrophils. In contrast, 32D/Y764F cells arrested in the G1 phase of the cell cycle within 24 hours and showed complete neutrophilic differentiation after 3 days of culture. This resulted in an average 30-fold reduction of neutrophil production as compared with the 32D/WT controls. Importantly, G-CSF-mediated activation of Shc, $p21^{Ras}$ and the induction of *c-myc* were severely reduced by substitution of Y764. These findings indicate that Y764 of G-CSF-R is crucial for maintaining the proliferation/differentiation balance during G-CSF-driven neutrophil development and suggest a role for multiple signaling mechanisms in maintaining this balance.

Introduction

Granulocyte colony-stimulating factor (G-CSF) is a 20- to 25-kD glycoprotein secreted by bone marrow stroma cells, macrophages, fibroblasts, and endothelial cells. G-CSF stimulates the proliferation and survival of myeloid progenitor cells, and their differentiation towards neutrophilic granulocytes (1,2). G-CSF-deficient mice show chronic neutropenia and a reduced granulopoietic response to infections, indicating that G-CSF plays an essential role in the regulation of granulopoiesis in both steady-state and stress conditions (3). The biological effects of G-CSF are mediated through a cell-surface receptor that is a member of the hematopoietin or class I cytokine receptor superfamily and that forms homodimeric complexes upon ligand binding (4,5).

Like other members of the hematopoietin receptor superfamily, the G-CSF receptor (G-CSF-R) lacks intrinsic tyrosine kinase activity, but activates cytoplasmic tyrosine kinases, in particular of the Jak family (2,5,6). Jaks associate with the membrane-proximal cytoplasmic region of the hematopoietin receptors and become activated upon ligand-induced receptor dimerization (7,8). Jak activation leads to tyrosine phosphorylation of the STAT (signal transducer and activator of

transcription) proteins, which form homodimers and/or heterodimers, translocate to the nucleus and activate target genes by interaction with specific DNA sequences. G-CSF stimulation results in the activation of Jak1, Jak2, STAT1, STAT3, and STAT5 (8-11).

Tyrosine kinase activity induced by G-CSF also results in the rapid phosphorylation of four conserved cytoplasmic tyrosines (Y) of the G-CSF-R protein (Y704, Y729, Y744, and Y764), located in the region distal to the conserved box 2 sequence (9,12). These phosphotyrosines form potential binding sites for signaling molecules that contain Src homology 2 (SH2) domains (13). For instance, Y704 of G-CSF-R, fitting the YXXQ consensus sequence for SH2-STAT3 binding, is involved in the recruitment and activation of STAT3 (14,15). Activation of Shc and SHP-2 (Syp), SH2-containing signaling intermediates of the $p21^{Ras}$ pathway, also requires recruitment via tyrosine residues of G-CSF-R (16). Notably, this depends on binding and activation of Jak kinases via the membrane-proximal region (17).

To accomplish neutrophilic differentiation in murine myeloid cell lines (32D, L-GM, or FDCP1), signals provided by the C-terminal region of G-CSF-R, spanning approximately 100 amino acids, are indispensible (18,19). This so-called differentiation domain of G-CSF-R contains three of the four cytoplasmic tyrosines (Y729, Y744, and Y764). To what extent the cytoplasmic tyrosines of G-CSF-R contribute to G-CSF-mediated proliferation and differentiation induction in myeloid cells has not been established.

In this study, we examined the consequences of tyrosine-to-phenylalanine (Y-to-F) substitutions in the cytoplasmic domain of G-CSF-R for the transduction of proliferation and differentiation signals in differentiation competent 32D cells. We show that all tyrosines can be replaced without affecting G-CSF-induced differentiation. Substitution of Y704, Y729, or Y744 had no effect on proliferation signaling. In contrast, mutant Y764F failed to support G-CSF-induced cell cycle progression from the G1 to the S phase, resulting in accelerated differentiation and significantly reduced net production of mature neutrophils. Strikingly, we found that activation of Shc, $p21^{Ras}$ and induction of *c-myc* are all mediated via Y764 of G-CSF-R, indicative of a potential role of these signaling molecules in maintaining the proliferation/differentiation balance in neutrophil development.

Materials and methods

G-CSF-R constructs and transfectants. Human *G-CSF-R* cDNA was cloned in the eukaryotic expression vector LNCX (20). Polymerase chain reaction techniques were used to generate the Y-to-F mutants Y704F, Y729F, Y744F, and Y764F, as described previously (16). A subline of the interleukin-3 (IL-3)-dependent murine myeloid cell line 32D (21), called 32D.C10 (22), was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng/ml of murine IL-3. The LNCX expression constructs were linearized by *Pvu*I digestion and transfected

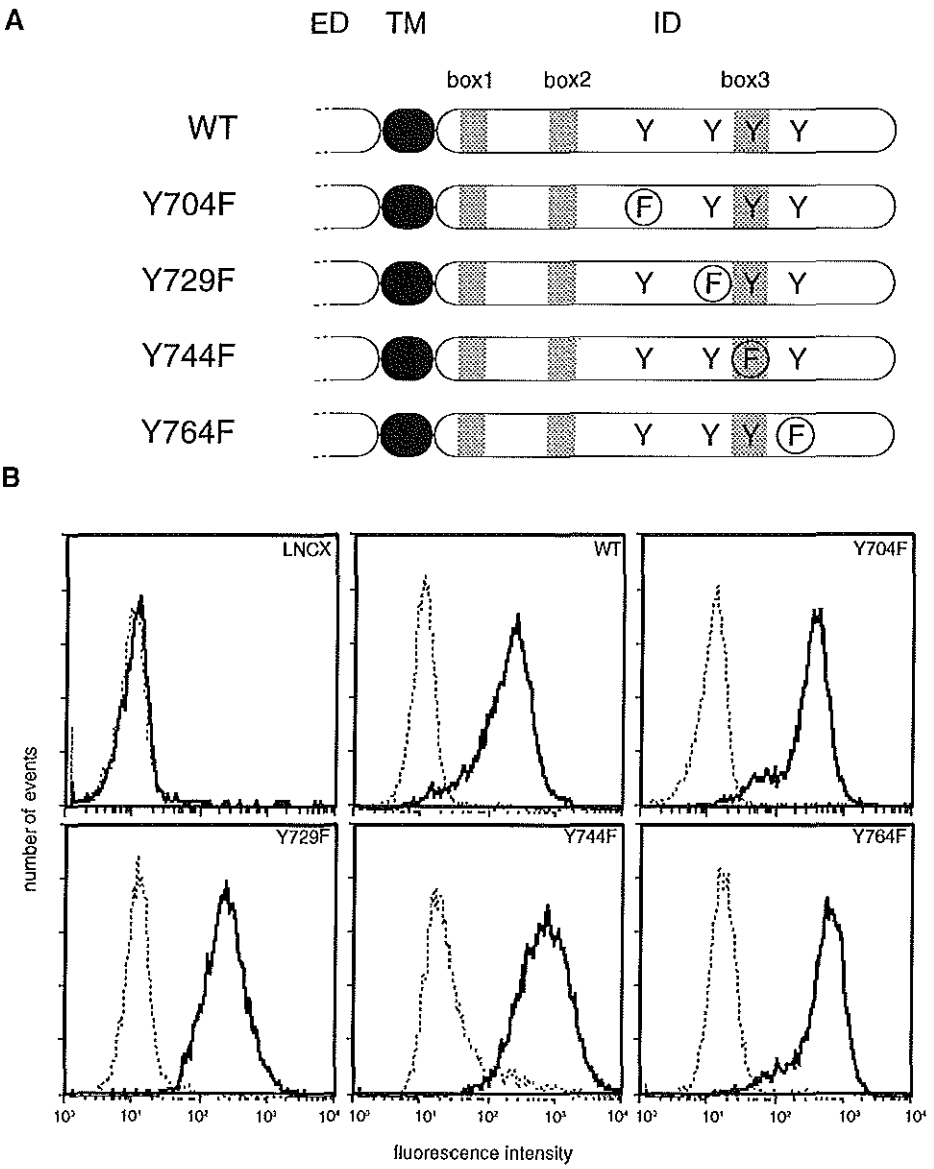

Figure 1. The G-CSF-R mutants. (A) Schematic diagram of the cytoplasmic domains of the G-CSF-R proteins. Boxes 1, 2, and 3 denote subdomains conserved in several members of the hematopoietin receptor superfamily. ED, extracellular domain; TM, transmembrane domain; ID, intracellular domain; Y, tyrosine; F, phenylalanine. (B) Flow cytometric analysis of G-CSF-R expression on 32D.C10 transfectants. Cells were either stained with biotinylated anti-G-CSF-R antibodies followed by PE-conjugated streptavidin (solid lines) or with PE-conjugated streptavidin alone (dotted lines).

into 32D.C10 cells by electroporation. After 48 hours of incubation, cells were selected with G418 (GIBCO-BRL, Breda, The Netherlands) at a concentration of 0.8 mg/ml. Multiple clones were expanded for further analysis. To determine G-CSF-R expression levels, cells were incubated at 4°C for 60 minutes with 10 µg/ml of biotinylated mouse antihuman G-CSF-R monoclonal antibody LMM741 (PharMingen, San Diego, CA). After washing, cells were treated at 4°C for 60 minutes with 5 µg/ml of phycoerythrin (PE)-conjugated streptavidin. Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA).

Cell proliferation and morphological analysis. To determine proliferation, cells were incubated at an initial density of 2 x 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 2 to 4 days, and the cell densities were adjusted to 2 to 4 x 10^5 cells/ml. Viable cells were counted on the basis of trypan blue exclusion. To analyze the morphologic features, cells were spun onto glass slides and examined after May-Grünwald-Giemsa staining.

Cell cycle analysis. For flow cytometric analysis of DNA content, cells were collected by centrifugation and resuspended in 0.1% sodium citrate containing 50 µg/ml of propidium iodide. The fluorescence of the stained cells was measured using a FACScan (Becton Dickinson). The Cell Fit program was used to determine the percentages of cells in the different phases of the cell cycle.

Shc immunoprecipitation. Preparation of cell lysates, immunoprecipitation, and Western blotting were performed as described (16). Anti-Shc antibodies (23) and antiphosphotyrosine antibodies 4G10 (Upstate Biotechnology Inc, Lake Placid, NY) were used.

$p21^{Ras}$ activation assay. Cells (1 x 10^7) were deprived of serum and growth factors and labeled by incubation for 3 hours in phosphate-free Dulbecco's modified Eagle's medium containing 100 µCi/ml of carrier-free [^{32}P]orthophosphate. Subsequently, the cells were stimulated for 5 minutes at 37°C with human G-CSF (1 µg/ml), murine IL-3 (1 µg/ml), or without factors (control). Cell lysis, immunoprecipitation of $p21^{Ras}$ with monoclonal antibody Y13-259, and thin layer chromatography were then performed as previously described (24). GTP binding to $p21^{Ras}$ was expressed as a percentage of total $p21^{Ras}$-bound guanine nucleotide (GTP + GDP) determined with a phosphorimager.

Analysis of c-myc expression. Cells were deprived of serum and growth factors for 4 hours and subsequently stimulated with human G-CSF (1 µg/ml) or murine IL-3 (1 µg/ml). At several time points, RNA was extracted from the cells using the Ultraspec-II RNA isolation system (Biotecx Laboratories Inc, Houston, TX). Agarose-formaldehyde gel electrophoresis and transfer to filters (Hybond; Amersham Life Sciences, Amersham, UK) was performed using standard procedures. As probes, a 1.4-kb *Eco*RI-*Hin*dIII fragment comprising the entire coding sequence of murine *c-myc*, and a 777-bp *Hin*dIII-*Eco*RI human *GAPDH* fragment (control) were ^{32}P-labeled by random priming (Boehringer, Mannheim, Germany).

Results

Experimental model. Tyrosine-to-phenylalanine substitution mutants of G-CSF-R are depicted in Figure 1A. Expression vectors encoding the various *G-CSF-R* cDNAs were introduced into a subline of the IL-3-dependent murine myeloid cell line 32D, called 32D.C10, that do not express endogenous G-CSF-R. In 32D.C10 cells transfected with the wild-type (WT) *G-CSF-R* cDNA, G-CSF induces transient proliferation followed by terminal neutrophilic differentiation after 8 to 11 days of culture (22). Expression levels of the different G-CSF-R proteins in the transfected 32D.C10 cells were determined by flow cytometry using G-CSF-R antibodies (Figure 1B). Several independent clones of each mutant with approximately equivalent G-CSF-R levels were selected for further analysis.

Mutation of Y764 of G-CSF-R inhibits proliferation but accelerates neutrophilic differentiation. To determine the abilities of WT and mutant G-CSF-R to induce proliferation and neutrophilic differentiation, 32D.C10 transfectants were switched from IL-3- to G-CSF-containing medium after extensive washing to remove residual IL-3. The experiments described below were performed and repeated on at least three independent clones of each mutant. Without IL-3 or G-CSF, all transfectants died within 1 day and showed no signs of neutrophilic differentiation. Parental 32D.C10 cells and cells transfected with empty LNCX vector also died within 1 day in G-CSF-containing medium. The 32D.C10 cells expressing WT G-CSF-R (32D/WT) proliferated in response to G-CSF for 6 to 8 days (Figure 2). After 8 to 11 days, 32D/WT cells developed into terminally differentiated neutrophils, showing an enlarged cytoplasm-to-nucleus ratio, neutrophilic cytoplasm, lobulated nuclei, granules, and expression of the murine neutrophil-specific surface antigen GR-1 (Figures 3A and B and data not shown). Similar results were obtained with

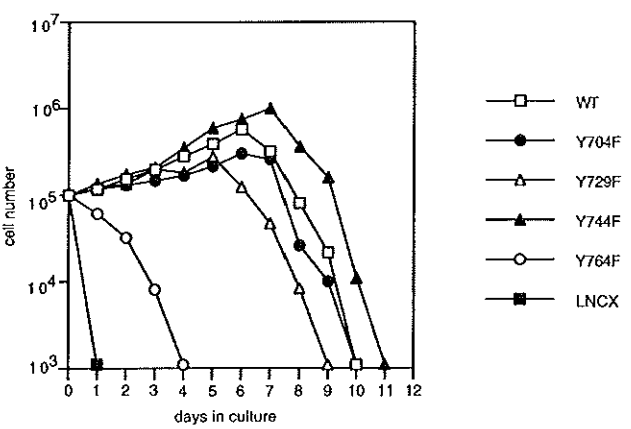

Figure 2. Proliferation of 32D.C10 transfectants in response to G-CSF. The numbers of viable cells were determined on the basis of trypan blue exclusion at the indicated times.

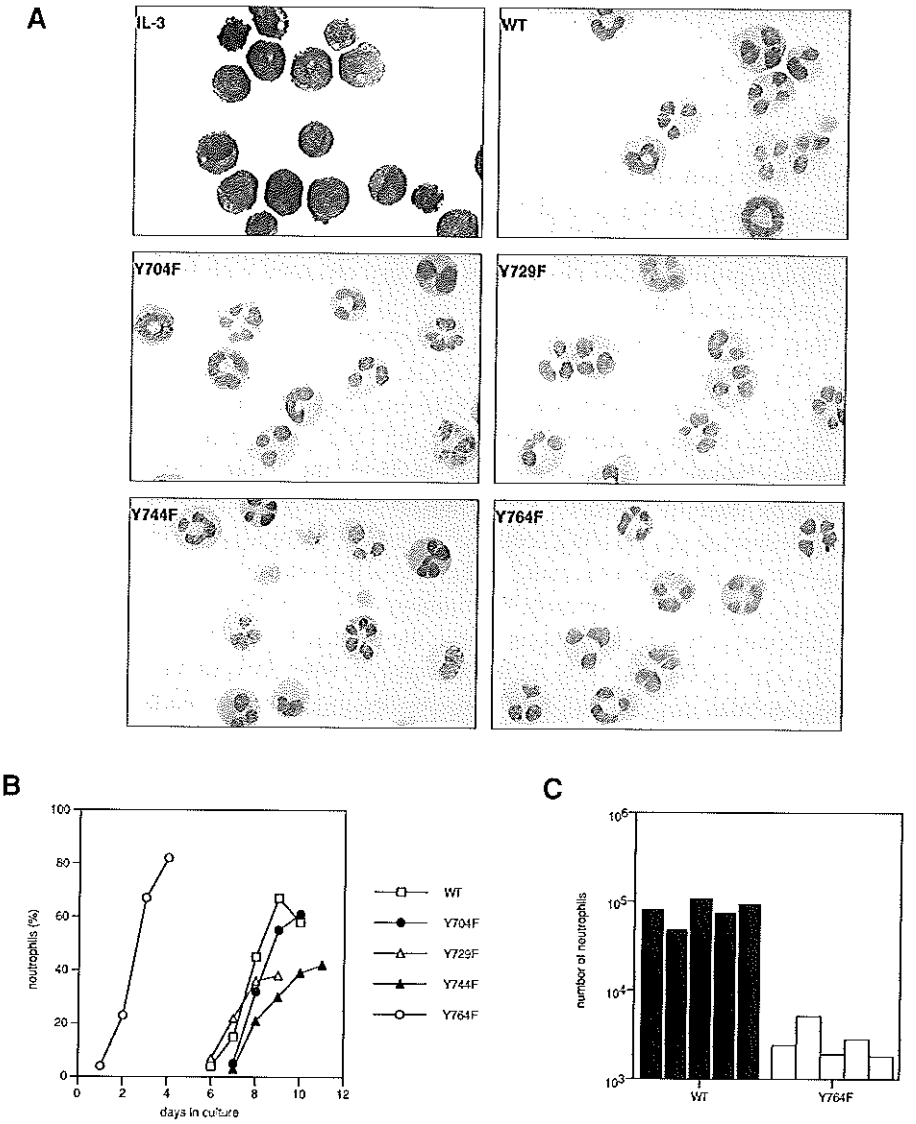

Figure 3. Neutrophilic differentiation of 32D.C10 transfectants in response to G-CSF. (A) Morphology of 32D.C10 transfectants maintained in IL-3-containing medium (IL-3) or cultured for 8 to 11 days (WT, Y704F, Y729F, Y744F) or for 3 days (Y764F) in the presence of G-CSF (May-Grünwald-Giemsa staining; original magnification x 630). (B) The percentages of terminally differentiated 32D.C10 transfectants cultured in G-CSF-containing medium. At least 200 cells were scored at the indicated times. (C) Net amount of mature neutrophils derived from 1 x 10^5 32D/WT and 32D/Y764F cells cultured in G-CSF-containing medium for 8 or 3 days, respectively. Data from five independent clones are shown.

32D/Y704F, 32D/Y729F, and 32D/Y744F cells. In contrast, 32D/Y764F cells did not proliferate in G-CSF-containing medium and showed terminal differentiation after 2 to 4 days, instead of 8 to 11 days. On average, this inappropriate balance of proliferation/differentiation resulted in a 30-fold reduced production of neutrophils as compared with 32D/WT cells (Figure 3C). Furthermore, ^{3}H-thymidine uptake assays after G-CSF stimulation showed that induction of DNA synthesis by mutant Y764F was severely reduced on day 1 and absent on day 2 of culture (data not shown). Stimulation of 32D/WT and 32D/Y764F cells with the combination of IL-3 and G-CSF resulted in proliferation rates similar to those obtained with IL-3 alone (Figure 4). In the presence of IL-3, G-CSF did not induce neutrophilic differentiation, indicating that IL-3-induced proliferation completely overrules the differentiation signaling by WT G-CSF-R and by mutant Y764F.

Y764 of G-CSF-R is essential for G-CSF-induced cell cycle progression from G1 to S phase. Cell cycle profiles of 32D/WT and 32D/Y764F cells were analyzed by flow cytometry (Figure 5). In the presence of IL-3, the majority of 32D/WT and 32D/Y764F cells were in S phase. After transfer to G-CSF-containing medium, the cell cycle distribution of 32D/WT cells changed only slightly during the first 48 hours, in agreement with the observation that the cells continued to proliferate at this stage of culture (Figure 2). In contrast, the number of 32D/Y764F cells in S phase had already significantly decreased 8 hours after the switch to G-CSF. After 48 hours, 87% of cells were in G1 and only 11% in S phase, with 35% of the total showing terminal neutrophilic differentiation. These results indicate that mutation of Y764 abrogates G-CSF-mediated cell cycle progression from the G1 to the S phase.

The WT G-CSF-R also induces accelerated differentiation in G1-arrested cells. To investigate whether accelerated neutrophilic differentiation in G-CSF-

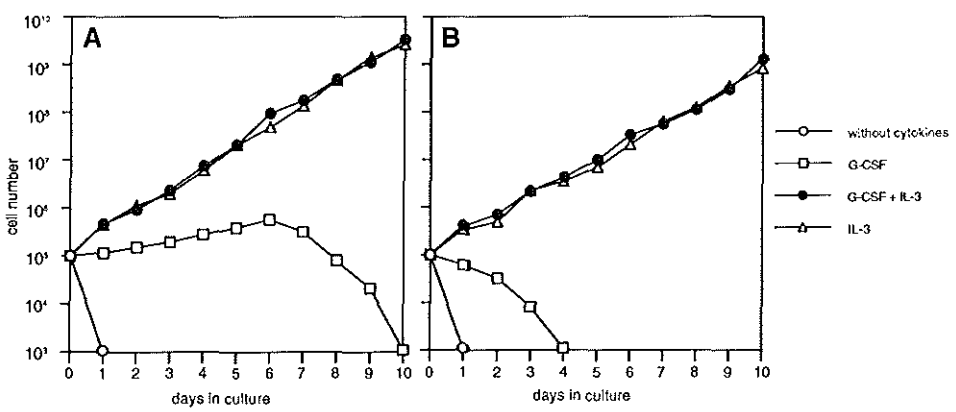

Figure 4. Proliferation of 32D/WT (A) and 32D/Y764F (B) cells in response to G-CSF and/or IL-3. The numbers of viable cells were determined on the basis of trypan blue exclusion at the indicated times.

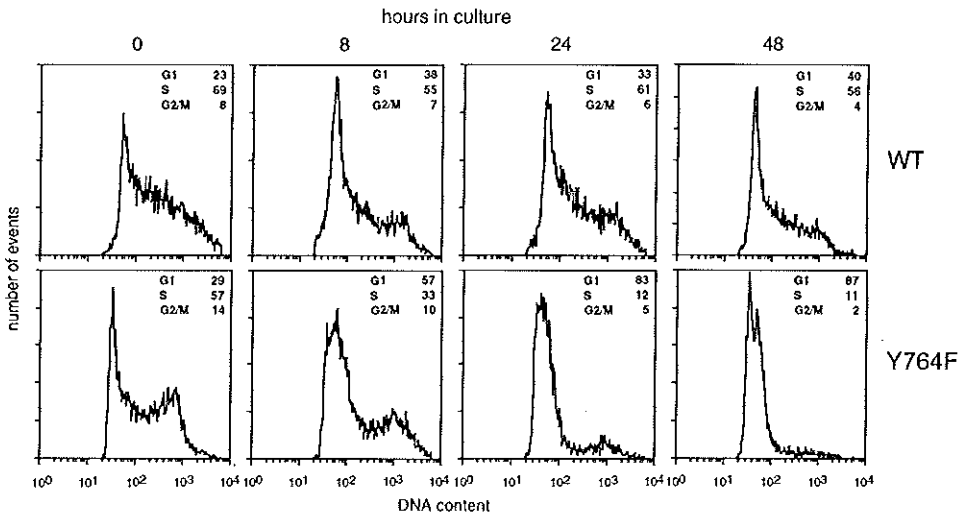

Figure 5. Cell cycle distribution of 32D/WT and 32D/Y764F cells at various time points after transfer to G-CSF-containing medium. The cells were stained with propidium iodide, and their DNA contents were analyzed by flow cytometry. The percentages of cells in the G1, S, and G2/M phases of the cell cycle are given in the upper right of each panel.

stimulated 32D/Y764F cells is the direct consequence of the lack of proliferation, we cultured 32D/WT cells in the presence of the cell cycle inhibitor cytosine arabinoside (Ara-C). Concentrations of Ara-C required to inhibit G-CSF- and IL-3-mediated proliferation with minimal cytotoxicity were 10^{-6} M and 10^{-5} M, respectively (data not shown). At these concentrations of Ara-C, cells cultured in IL-3- or G-CSF-containing medium gradually lost viability and died after 4 to 5 days (Figure 6A). Cell cycle analysis showed that the cells were arrested in G1 (Figure 6B). In the G-CSF-containing cultures, terminal neutrophilic differentiation occurred after 2 to 4 days in the presence and after 7 to 9 days in the absence of Ara-C (Figures 6C and D). Thus, 32D/WT cells arrested in G1 by Ara-C also show accelerated differentiation in response to G-CSF. In contrast, IL-3 did not induce neutrophilic differentiation in G1-arrested 32D/WT cells (Figure 6C). Essentially similar results were obtained using hydroxyurea as a cell cycle inhibitor (data not shown).

Activation of Shc and p21^{Ras} and expression of c-myc are mediated via Y764 of G-CSF-R. We previously showed in lymphoid BAF3 cells that Shc/GRB2 and SHP-2/GRB2 complexes, implicated in activation of p21^{Ras} by a variety of receptor systems, are both activated by G-CSF-R (16). Multiple tyrosines in G-CSF-R mediate the formation of SHP-2/GRB2 complexes, but Shc activation and Shc/GRB2 association critically depend on Y764 of G-CSF-R (16). We first confirmed that Y764 is also essential for G-CSF-induced Shc activation in myeloid 32D.C10 transfectants

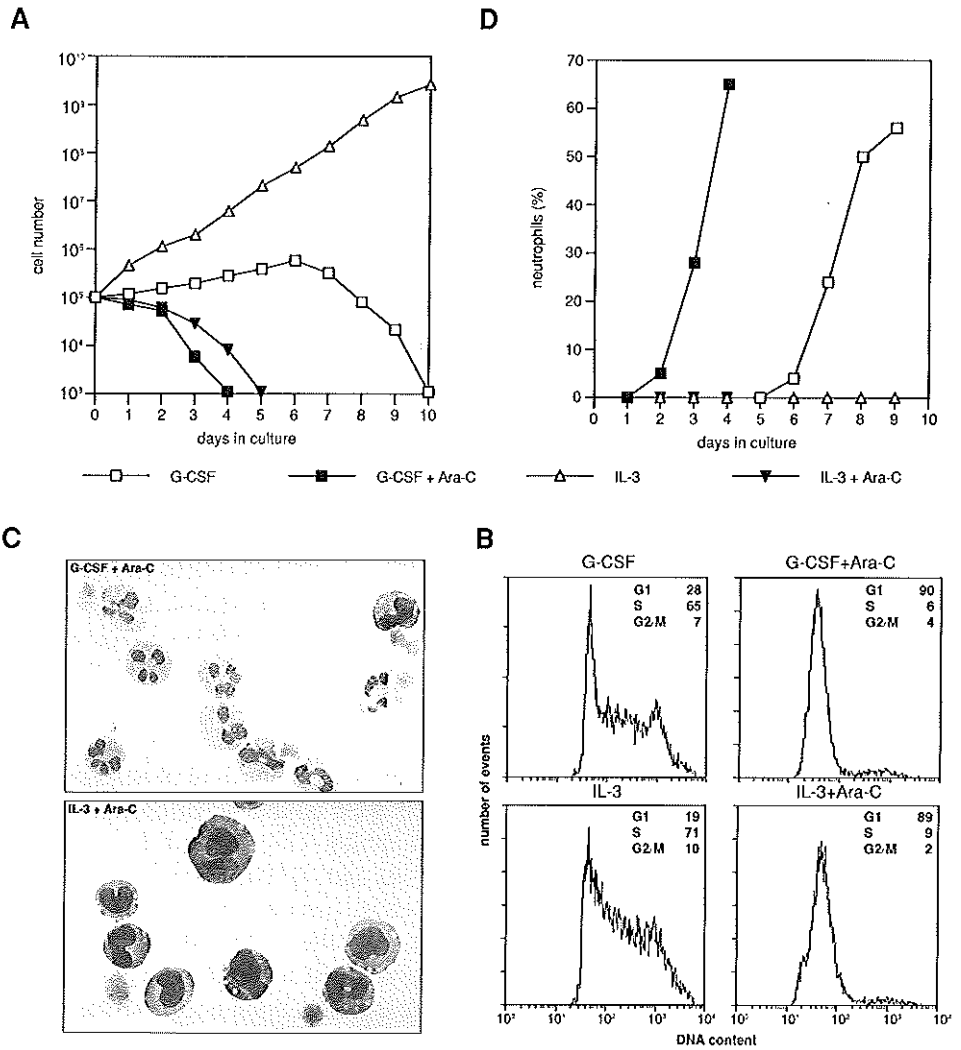

Figure 6. Effect of Ara-C on proliferation and differentiation of 32D/WT cells. (A) G-CSF- and IL-3-dependent proliferation of 32D/WT cells in the absence or presence of Ara-C. The numbers of viable cells were determined on the basis of trypan blue exclusion at the indicated times. (B) Cell cycle distribution of 32D/WT cells cultured for 3 days in G-CSF- or IL-3-containing medium in the absence or presence of Ara-C. The percentages of cells in the G1, S, and G2/M phases of the cell cycle are given in the upper right of the panels. (C) Morphology of 32D/WT cells cultured for 3 days in G-CSF- or IL-3-containing medium in the presence of Ara-C (May-Grünwald-Giemsa staining; original magnification x 630). (D) The percentages of terminally differentiated 32D/WT cells cultured in G-CSF- or IL-3-containing medium in the absence or presence of Ara-C. At least 200 cells were scored at the indicated times.

(Figure 7). Ras-loading assays indicated that activation of WT G-CSF-R resulted in an approximately eightfold increase of $p21^{Ras}$-GTP as compared with nontreated controls (Figure 8, middle panel). In contrast, activation of mutant Y764F induced a marginal increase of $p21^{Ras}$-GTP over background levels (Figure 8, right panel). Control cells transfected with empty LNCX vector showed no activation of $p21^{Ras}$ in response to G-CSF (Figure 8, left panel).

Because it has recently been established that Shc is involved in the induction of *c-myc* expression via a novel GRB2- and $p21^{Ras}$-independent mechanism (25,26), we also investigated the ability of G-CSF-R mutant Y764 to activate *c-myc*. We found that G-CSF-induced expression of *c-myc* was severely reduced in 32D/Y764F cells as compared with 32D/WT cells, whereas responses to IL-3 had not changed (Figure 9).

Discussion

Previously, we have investigated the contribution of the cytoplasmic tyrosine residues of G-CSF-R to signaling using BAF3 cell transfectants expressing Y-to-F substitution mutants (15,16). These studies provided information on the specific involvement of these tyrosines in the activation of signaling substrates of the Jak/STAT and $p21^{Ras}$ signaling pathways. However, replacement of the tyrosines did not affect the proliferation signaling abilities of G-CSF-R. This was not unexpected, because earlier work had shown that truncated forms of G-CSF-R, which lack all tyrosine residues and which fail to mediate proliferation in myeloid 32D or L-GM cells, still efficiently transduced proliferation signals in BAF3 cells (18,27). Apparently, proliferation control mechanisms mediated via regions C-terminal of the box 2 consensus domain are bypassed in BAF3 cells. This, combined with their inability to differentiate towards the myeloid lineage, makes BAF3 cells not suitable for studying the coupled proliferation/differentiation response to G-CSF.

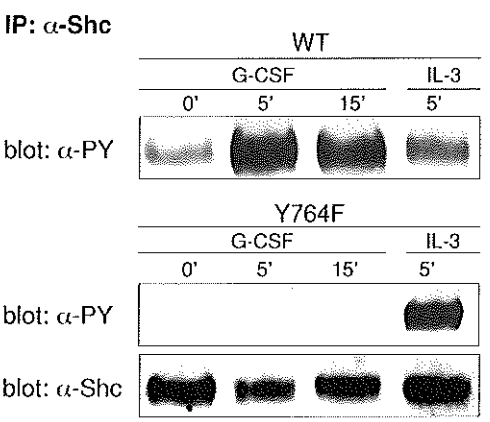

Figure 7. Shc immunoprecipitation on lysates from 32D/WT and 32D/Y764F cells. Serum- and growth factor-deprived cells were stimulated for 5 or 15 minutes at 37°C with G-CSF or IL-3. The blots were hybridized with antiphosphotyrosine antibodies and reprobed with anti-Shc antibodies to confirm equal loading of Shc.

We reported here on four observations relevant to the balanced proliferation and differentiation response to G-CSF in differentiation competent 32D cells. [1] A single tyrosine (Y764) of human G-CSF-R plays a key role in maintaining this balance by activation of mechanisms that control cell cycle progression at the level of G1 to S transition. [2] Shc, $p21^{Ras}$, and Myc are activated via this tyrosine. [3] Inhibition of proliferation does not prevent but accelerates terminal differentiation. [4] Enforced cell cycle arrest does not induce terminal neutrophilic differentiation in the absence of G-CSF.

Activation of $p21^{Ras}$ via Y764 of G-CSF-R is likely to play a major role in G-CSF-induced proliferative responses. Previous studies provided evidence for the involvement of $p21^{Ras}$ in IL-3- or G-CSF-induced cell cycle progression in myeloid cells. Expression of a dominant-inhibitory mutant of $p21^{Ras}$ blocked G-CSF-mediated proliferation of 32Dcl3 cells and caused a G1 arrest (28). Further, enforced expression of constitutively active Raf-1, a downstream target of $p21^{Ras}$, promoted cell cycle progression, whereas *c-raf* antisense oligonucleotides inhibited G-CSF-induced proliferation of 32Dcl3 cells (29,30).

In addition to $p21^{Ras}$, Myc has been implicated in the control of proliferation of myeloid cells. Depending on the cell system, enforced expression of *c-myc* either induced cell cycle progression or premature apoptosis, indicative of the dualistic role

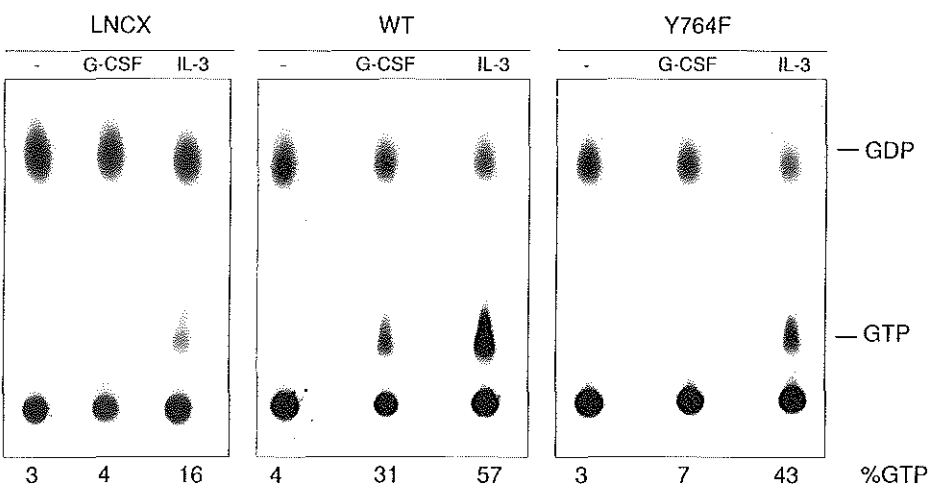

Figure 8. Activation of $p21^{Ras}$ in 32D.C10 transfectants. Serum- and growth factor-deprived cells were labeled with [^{32}P]orthophosphate and subsequently stimulated for 5 minutes at 37°C without factor (-), with G-CSF, or with IL-3. Cells were lysed and $p21^{Ras}$ was collected by immunoprecipitation. Nucleotides bound to $p21^{Ras}$ were eluted and separated by thin-layer chromatography. The positions of the GTP and GDP standards are indicated. The level of $p21^{Ras}$-GTP, expressed as a percentage of total nucleotide bound to $p21^{Ras}$ (GTP + GDP), is given under each lane.

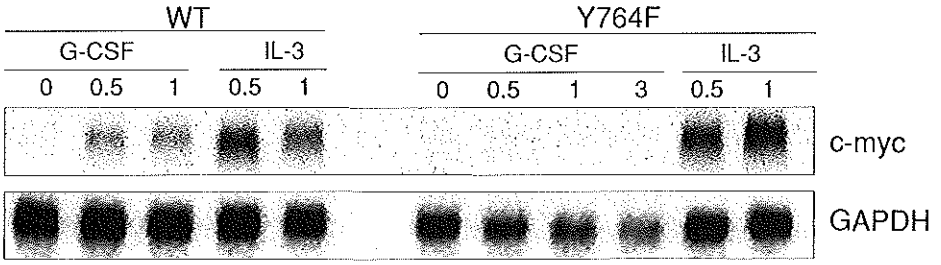

Figure 9. Induction of *c-myc* expression in 32D/WT and 32D/Y764F cells. Serum- and growth factor-deprived cells were stimulated for 0.5, 1, or 3 hours at 37°C with G-CSF or IL-3. Total RNA (10 μg) was analyzed by Northern blot hybridization using a ^{32}P-labeled *c-myc* probe. The blot was reprobed with *GAPDH* to confirm equal loading.

played by Myc in controlling these processes (31-33). Conversely, inhibition of *c-myc* with antisense oligonucleotides inhibited proliferation and permitted differentiation in HL-60 cells (34). Importantly, $p21^{Ras}$ and Myc have recently been found to collaborate in activation of cyclinE/cdk2 complexes (35). Thus, it is likely that the activation of $p21^{Ras}$ and induction of *c-myc* expression via Y764 of G-CSF-R both play a crucial role in the control of cell cycle progression and thereby in the timing of neutrophilic differentiation. Finally, Y763 of murine G-CSF-R (analogous to human Y764) has been implicated in yet another signaling mechanism, i.e. involving activation of JNK/SAPK (36). Although the significance of this finding for the regulation of cell cycle progression is not yet clear, it underscores the significance of this tyrosine residue for G-CSF-R function.

The docking protein Shc is activated by different members of the hematopoietin receptor superfamily. By virtue of its binding via Y317 to GRB2, Shc has been implicated primarily in the activation of $p21^{Ras}$, Raf-1, and ERK1/ERK2 (37). Recently, a second mechanism of Shc-mediated signaling was discovered (25,26). This route requires residues Y239/240 instead of Y317 of the Shc protein and involves expression of the *c-myc* gene. Intriguingly, depending on the receptor type and cell system, distinct cellular responses have been associated with Shc. For instance, Inhorn *et al.* (38) showed that activation of Shc by granulocyte-macrophage colony-stimulating factor (GM-CSF) is mediated via Y750 of the GM-CSF receptor β chain and that site-directed mutagenesis of this tyrosine results in reduced cellular viability of BAF3 transfectants in response to GM-CSF. On the other hand, overexpression of Shc proteins in GM-CSF-dependent TF-1 cells increases the proliferative response to GM-CSF, in agreement with a role for Shc in mitogenic signaling (39). Finally, Y599 of the c-Mpl receptor has been implicated in Shc phosphorylation and macrophage differentiation in WEHI3B-D^+ cells (40). With regard to the role of Shc in G-CSF-R function, our data are most consistent with a model in which two pathways, Shc to $p21^{Ras}$ and Shc to Myc, both activated via Y764

of G-CSF-R, are involved in the regulation of G-CSF-driven cell cycle progression, but are not required for execution of the neutrophilic differentiation program.

Previously, we detected point mutations in the *G-CSF-R* gene in patients with severe congenital neutropenia who showed disease progression to acute myeloid leukemia (22,41). These mutations introduce premature stop codons between amino acids 715 and 731 and result in the deletion of the C-terminal cytoplasmic region of the receptor that is essential for the induction of neutrophilic differentiation (18,19,22). Contrary to the transient proliferative responses provided by WT G-CSF-R, activation of G-CSF-R mutants lacking the C-terminal differentiation domain resulted in sustained and enhanced proliferation in myeloid cells. Paradoxically, such truncated G-CSF-R were found to activate $p21^{Ras}$ and *c-myc* at levels comparable to WT G-CSF-R, despite the fact that they lack Y764 (De Koning *et al.*, unpublished data). The mechanisms by which this is achieved are not yet clear, but do not appear to involve Shc, because Shc is not detectably activated by these truncated forms of G-CSF-R (16). Most likely, activation of $p21^{Ras}$ is mediated via SHP-2/GRB2 complexes that bind to Y704 (16). As yet, we have no data to explain why these alternative mechanisms are not activated by G-CSF-R mutant Y764. One possibility is that this is due to negative interference, caused by the configuration of full-length G-CSF-R.

We found that mutation of Y704, Y729, Y744, or Y764 did not affect the ability of G-CSF-R to transduce differentiation signals in 32D.C10 cells. In contrast, Yoshikawa *et al.* (12) reported that substitution of Y703 or Y728 of the murine G-CSF-R (analogous to human Y704 and Y729, respectively) prevented G-CSF-induced neutrophilic differentiation in L-GM-1 transfectants. It is relevant to note that, although the cells used by Yoshikawa *et al.* (12) showed expression of myeloperoxidase transcripts in response to G-CSF, cytologically the cells did not show the characteristics of terminally mature neutrophils as observed in our study. A further complication is that the experiments with the L-GM-1 cells were performed with pools of five or six clones for each mutant (12). In such pools, outgrowth of one or more clones with growth advantage can easily take place. This could result in the selective loss of G-CSF-mediated growth arrest and, consequently, the absence of neutrophilic differentiation, as observed by Yoshikawa *et al.* (12) in the pools of the Y703F and Y728F transfectants. In M1 cells, differentiation in response to G-CSF was reduced by mutation of Y744 of G-CSF-R and, to a lesser extent, by mutation of Y704 and Y729 (42). A crucial difference between the M1 and the 32D.C10 cell models is that M1 cells expressing WT G-CSF-R differentiate into macrophages, instead of neutrophils. Obviously, specific signaling molecules required for macrophage differentiation may differ from those involved in neutrophilic differentiation. Therefore, the tyrosine residues of G-CSF-R essential for macrophage differentiation in the M1 model need not have any relevance to neutrophilic differentiation. More recently, we observed that G-CSF also induces complete neutrophilic differentiation in 32D cells expressing the triple mutant Y729/744/764F,

indicating that the tyrosines in the C-terminal part of G-CSF-R are not essential for differentiation induction (De Koning *et al.*, unpublished data).

Our findings strongly suggest that differentiation induction by WT G-CSF-R does not require active cell cycling. Rather, downmodulation of G-CSF-mediated proliferation and accumulation in G1 appeared a prerequisite for neutrophilic differentiation (Figures 5 and 6) (43,44). Identification of the signaling substrates that are essential for differentiation and understanding of how these molecules influence the signals provided via Y764 of G-CSF-R will shed further light on how the balance between proliferation and differentiation is maintained at progressive stages of neutrophil development. This knowledge may provide important clues as to how this balance may be affected in diseases characterized by a disturbed production of neutrophils, such as severe congenital neutropenia and acute myeloid leukemia.

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CHAPTER 6

STAT3-controlled expression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ during G-CSF-induced growth arrest of myeloid cells

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Submitted

Abstract

The signal transducer and activator of transcription STAT3 has been implicated in the control of granulocyte colony-stimulating factor (G-CSF)-induced neutrophilic differentiation as well as interleukin-6-induced macrophage development, but the underlying molecular mechanism(s) have remained unclear. Here, we provide evidence to suggest that STAT3 is not essential for G-CSF-induced differentiation itself, but induces a growth arrest that is a prerequisite for myeloid precursor cells to proceed with differentiation. The splice variant STAT3β, previously shown to be a dominant-negative form of STAT3 on certain promoters, did not affect cell growth. Interestingly, expression of the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$, but not of $p21^{WAF1/CIP1}$, increased during G-CSF stimulation of myeloid cells on both protein as well as on mRNA level. An oligonucleotide derived from the promoter region of p27 containing a putative STAT-binding site shifted with STAT3 in electrophoretic mobility shift assays. Dominant-negative STAT3 reduced G-CSF-induced p27 promoter activity in luciferase reporter assays and interfered with the expression of both p27 mRNA and p27 protein. Furthermore, bone marrow and spleen mononuclear cells of p27-deficient mice showed an increased proliferative capacity in response to G-CSF. These results suggest that STAT3-controlled cell cycle exit of myeloid precursors is mediated via direct upregulation of the cdk inhibitor p27.

Introduction

Cytokine receptors lack intrinsic tyrosine kinase activity but activate cytoplasmic tyrosine kinases, in particular of the Janus kinase (Jak) family (1,2). Jaks associate with the membrane-proximal cytoplasmic region of the receptors and become activated upon ligand-induced receptor dimerization (3,4). Jak activation leads to phosphorylation of the STAT (signal transducer and activator of transcription) proteins on a conserved tyrosine residue, just C-terminal to the Src homology 2 (SH2) domain (5). Subsequently, STATs dimerize by phosphotyrosine/SH2 interactions, translocate to the nucleus and activate target genes by interaction with specific DNA sequences. Recently, a role for STATs in the control of cell growth and development has been demonstrated in a variety of cell systems. For instance, it was shown that STAT5 is involved in interleukin-3 (IL-3)-induced proliferation of BAF3 cells (6). Activation of STAT3 was linked to IL-6-induced growth arrest and macrophage differentiation of M1 cells (7), IL-10-induced inhibition of macrophage proliferation (8), and granulocyte colony-stimulating factor (G-CSF)-induced neutrophilic differentiation (9). However, the underlying mechanism(s) of STAT-mediated control of growth and differentiation are still largely unclear.

G-CSF stimulates proliferation and survival of myeloid progenitor cells and their differentiation towards neutrophilic granulocytes (1,10). G-CSF-deficient mice show

chronic neutropenia and a reduced granulopoietic response to bacterial infections, indicating that G-CSF plays an essential role in the regulation of granulopoiesis in both steady-state and stress conditions (11). The biological effects of G-CSF are mediated through a cell-surface receptor (G-CSF-R) that belongs to the hematopoietin or class I cytokine receptor superfamily and that forms homodimeric complexes upon ligand binding (12,13). G-CSF activates STAT1, STAT3, and STAT5 (14,15). Whereas the membrane-proximal cytoplasmic region of G-CSF-R is sufficient for activation of STAT1 and STAT5, activation of STAT3 requires the membrane-distal C-terminal part of the receptor (15,16). The G-CSF-R C-terminus contains four conserved tyrosine residues (Y704, Y729, Y744, and Y764) and comprises a region that is implicated in the control of neutrophilic differentiation (17,18). Y704, located within a YXXQ consensus sequence for STAT3-SH2 binding, is involved in STAT3 activation by G-CSF-R. However, other domains within the G-CSF-R C-terminus also mediate STAT3 activation via as yet unknown interactions (16).

In this study, we show that increased levels of activated STAT3 are not required for induction of differentiation per se, but have a role in G-CSF-induced growth arrest via induction of the cyclin-dependent kinase (cdk) inhibitor $p27^{Kip1}$. Thus far, p27 has been implicated mainly in transforming growth factor β-, cyclic AMP-, tamoxifen-, or cell-cell contact-mediated growth arrest via inhibition of cyclin A/cdk2, cyclin D/cdk4, and cyclin E/cdk2 complexes (19-21). We provide evidence that p27 is under the direct transcriptional control of STAT3. Finally, we demonstrate that the STAT3 splice variant STAT3β, previously associated with myeloid differentiation, is not involved in G-CSF-induced responses.

Materials and methods

Cell culture, constructs, and transfectants. 32D.C8.6, a subline of the IL-3-dependent murine myeloid cell line 32D (22), was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng/ml of murine IL-3. The pBabe expression vector (23) encoding the human *G-CSF-R* cDNA was introduced into 32D.C8.6 cells by electroporation. Subsequently, cells were selected with puromycin (Sigma, Zwijndrecht, The Netherlands) at a concentration of 1 μg/ml. The G-CSF-R expression levels in multiple clones were determined by flow cytometry as previously described (24). Various *STAT3* cDNAs cloned into the pCAGGS-Neo or pLNCX expression vector were transfected into a 32D.C8.6 clone overexpressing human G-CSF-R. The pCAGGS-Neo constructs encoding wild-type (WT) hemagglutinin peptide (HA)-tagged murine *STAT3* cDNA or the dominant-negative mutants HA-STAT3F and HA-STAT3D were used (kindly provided by Drs. K. Nakajima and T. Hirano). The tyrosine residue at position 705 was substituted for phenylalanine in HA-STAT3F, whereas glutamic acids 434 and 435 were replaced with alanines in HA-STAT3D (7). The *STAT3β* cDNA (25) was cloned into the pLNCX vector. After transfection, cells were selected with G418 (GIBCO-BRL,

Breda, The Netherlands) at a concentration of 0.8 mg/ml. Several independent clones were expanded for further analysis.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared as previously described (16). Extracts of 2 x 10^6 cells were incubated for 20 minutes at room temperature with 0.2 ng of ^{32}P-labeled double-stranded oligonucleotide (5 to 10000 cpm) 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, 8% glycerol). The following oligonucleotides were used: m67 (5' CATTTCCCGTAAATC 3'), a high-affinity mutant of the sis-inducible element (SIE) of the human *c-fos* gene (26); β-casein (5' AGATTTCTAGGAATTCAATCC 3'), the STAT5-binding site of the bovine β-casein promoter (27); p27 (5' AATTTCCTGTAACAT 3'), a potential STAT-binding site in the $p27^{Kip1}$ promoter located at position -1585 (28); and p21SIE2 (5' GATCCTTTCTGAGAAATGG 3'), a potential STAT-binding site in the $p21^{WAF1/CIP1}$ promoter located at nucleotide -2540 from the TATA promoter site (29). The DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gels containing 5% glycerol in 0.25 x TBE. The gels were dried and subsequently analyzed by autoradiography. For competition analysis, nuclear extracts were preincubated for 20 minutes with 500-fold excess of unlabeled double-stranded oligonucleotide before the addition of ^{32}P-labeled probe. For supershift analysis, nuclear extracts were preincubated for 2 hours with 2 µg of anti-STAT3 (raised against amino acids 750-769; Santa Cruz Biotechnology Inc, Santa Cruz, CA), anti-STAT1α (raised against amino acids 716-739; Santa Cruz), anti-STAT1αβ antibodies (raised against amino acids 688-710 mapping within the C-terminal sequence common to STAT1α and STAT1β; Santa Cruz), or anti-STAT5 antibodies (provided by Dr. T. Decker, Vienna, Austria) before the addition of ^{32}P-labeled oligonucleotide.

Immunoprecipitation and Western blotting. Preparation of cell lysates, immunoprecipitation, and Western blotting were performed as described (30). Anti-STAT3 (raised against amino acids 750-769; Santa Cruz), anti-STAT3 (raised against amino acids 1-175; Transduction Laboratories Inc, Lexington, KY), anti-HA (Boehringer, Mannheim, Germany), and anti-p27 antibodies (Transduction Laboratories) were used.

Cell proliferation and morphological analysis. To determine proliferation, cells were incubated at an initial density of 3 x 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 2 to 4 days, and the cell densities were adjusted to 3 x 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.

Luciferase assay. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% FCS. For transient transfection, cells were plated into 12-well plates at a density of 9 x 10^4 cells/well and cultured for 24 hours. Subsequently, cells were transfected by the DEAE-dextran method with

250 ng of $p27^{Kip1}$-pGL-2, a reporter construct containing the p27 promoter (-1609 to +178) upstream of the *luciferase* gene (28), 250 ng of pLNCX-G-CSF-R, an expression vector containing the human *G-CSF-R* cDNA (17), and 250 ng of pCAGGS constructs encoding the various *STAT3* cDNAs. Although the STAT3 variants were tested in several ratios, the total amount of pCAGGS plasmid was kept constant at 250 ng in each transfection. Cells were incubated with DNA/DEAE-dextran precipitates for 30 minutes, washed and cultured in 10% FCS/DMEM medium supplemented with 100 ng/ml of human G-CSF, 200 ng/ml of cholera toxin, 500 nM forskolin, 500 µM dibutyryl-cAMP, or without factors. After 48 hours, cells were lysed in luciferase lysis buffer (25 mM Trisphosphate [pH 7.8], 8 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, 15% glycerol) and assayed for luciferase activity on a Biocounter M2500 luminometer (Lumac, Landgraaf, The Netherlands) using an equal volume of luciferin solution (1 mM luciferin, 1 mM ATP, 8 mM $MgCl_2$) as a substrate.

Northern blotting. RNA was extracted from cells using the Ultraspec-II RNA isolation system (Biotecx Laboratories Inc, Houston, TX). Agarose-formaldehyde gel electrophoresis and transfer to filters (Hybond; Amersham Life Sciences, Amersham, UK) was performed using standard procedures. As probes, murine $p27^{Kip1}$ (888-bp *Not*I fragment) and murine *GAPDH* (777-bp *Hind*III-*Eco*RI fragment) were ^{32}P-labeled by random priming (Boehringer, Mannheim, Germany). For quantification, filters were exposed to phosphorimager screens and analyzed with ImageQuant software (Molecular Dynamics).

In vitro colony assays. The generation of p27-deficient mice (kindly provided by Dr. J. Roberts) has been previously described (31). Femurs, tibias, and spleens of 6 to 12 month old male knockout ($p27^{-/-}$) and wild-type ($p27^{+/+}$) mice were removed aseptically. To obtain bone marrow cell suspensions, femurs and tibias were crushed in a mortar in HBSS/10% FCS. Both spleen and bone marrow cells were passed through a 70 µm sieve, spun down, and resuspended, resulting in mononuclear suspensions containing 98% to 99% viable cells as determined by trypan blue exclusion. Subsequently, cells were incubated in a cell culture flask in a humidified atmosphere containing 5% CO_2 at 37°C for 1 hour. Nonadherent cells were harvested, and 3 x 10^4 bone marrow or 3 x 10^5 spleen mononuclear cells were plated in triplicate in 35 mm Petri dishes in 1 ml of methylcellulose medium containing 30% fetal bovine serum, 1% bovine serum albumin, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, without additional growth factors, or with increasing concentrations of G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-3. Colonies containing at least 30 cells were counted on day 7 of culture. To analyze the morphological features and to determine the average cell number per colony, cultures were harvested and washed extensively to remove methylcellulose. Cells were counted, spun onto glass slides, and examined after May-Grünwald-Giemsa staining.

Results

G-CSF induces sustained STAT3 activation in 32D cells undergoing proliferation followed by neutrophilic differentiation. IL-3-dependent 32D cells have an immature myeloblastic morphology. 32D transfectants expressing the human G-CSF-R respond to G-CSF by transient proliferation, followed by terminal neutrophilic differentiation after 7 to 10 days of culture (24). To investigate STAT activation during progressive differentiation, nuclear extracts were prepared daily

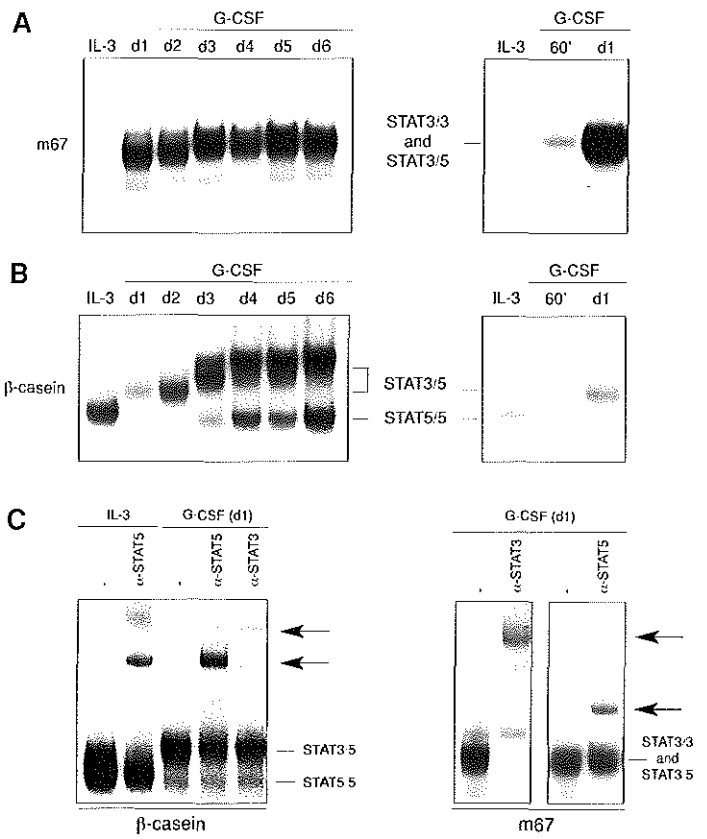

Figure 1. EMSA of nuclear extracts from 32D cells expressing G-CSF-R. Cells were switched from IL-3- to G-CSF-containing medium after extensive washing to remove residual IL-3 and incubated at 37°C for the times indicated. Nuclear extracts were prepared and incubated with ^{32}P-labeled double-stranded m67 (A) or β-casein (B) oligonucleotides. For supershift analysis, nuclear extracts were preincubated without (-) or with the indicated antibodies before the addition of ^{32}P-labeled oligonucleotides (C). The supershifted complexes are indicated by arrows.

after switching the cells from IL-3- to G-CSF-containing medium. EMSAs with m67 oligonucleotides showed that STAT3- and/or STAT1-containing complexes were not activated in 32D cells proliferating in IL-3-containing medium. In contrast, G-CSF induced sustained activation of STAT3 homodimers (Figure 1A). In addition, some STAT1-STAT3 heterodimers, but no STAT1 homodimers, were formed. EMSAs with β-casein oligonucleotides showed that the level of STAT5 homodimers was not significantly altered by switching the cells from IL-3- to G-CSF-containing medium (Figure 1B). Interestingly however, several other presumably heteromeric nuclear complexes that bound to β-casein oligonucleotides were induced by G-CSF. Indeed, these complexes could be supershifted with both anti-STAT3 and anti-STAT5 antibodies, whereas addition of anti-STAT1 antibodies did not affect the mobility of the complexes. Furthermore, addition of anti-STAT5 also in part supershifted complexes that bound to m67 oligonucleotides (Figure 1C), indicating that G-CSF induces the formation of STAT3-STAT5 heteromeric complexes with affinity for both m67 and β-casein oligonucleotides. The mobility of the STAT3-STAT5 complexes slightly decreased after 3 to 4 days of G-CSF stimulation.

Establishment of 32D transfectants stably expressing dominant-negative STAT3. To examine the role of STAT3 in G-CSF-mediated responses, we introduced expression vectors encoding wild-type (WT) hemagglutinin (HA)-tagged STAT3 (HA-STAT3WT) or two different dominant-negative (DN) STAT3 mutants (HA-STAT3F and HA-STAT3D) into the G-CSF-R expressing 32D cells. Expression

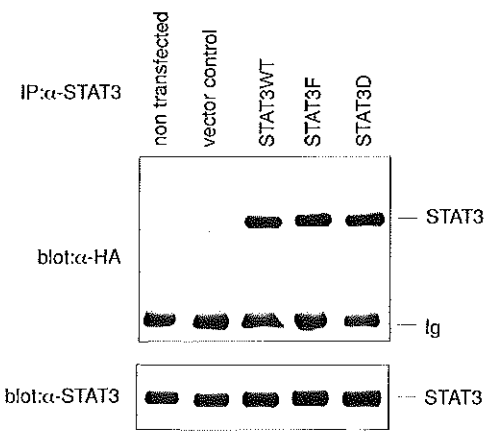

Figure 2. Expression of STAT3 variants in 32D/G-CSF-R cells. In STAT3F, tyrosine residue at position 705 essential for STAT dimer formation was substituted for phenylalanine. In STAT3D, glutamic acids 434 and 435 important for DNA binding were replaced by alanines. STAT3 immunoprecipitation on lysates of 32D transfectants cultured in IL-3-containing medium. Immunoprecipitates were subjected to Western blot analysis using anti-HA antibodies. Blots were reprobed with anti-STAT3 antibodies (against amino acids 750-769). Ig, immunoglobulins.

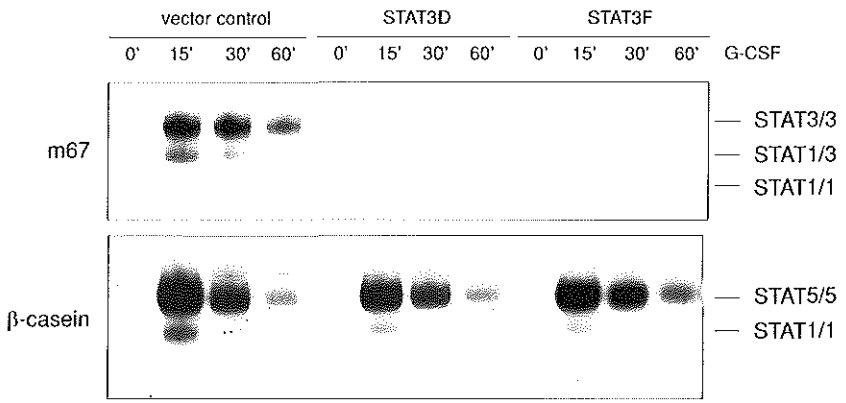

Figure 3. EMSA of nuclear extracts from 32D transfectants expressing STAT3 variants. Serum- and growth factor-deprived cells were incubated at 37°C with G-CSF (100 ng/ml) for the times indicated. Nuclear extracts were prepared and incubated with ^{32}P-labeled double-stranded m67 or β-casein oligonucleotides.

levels of the STAT3 variants were determined in multiple independent transfectants by immunoprecipitation with anti-STAT3 antibodies, followed by Western blotting with anti-HA antibodies (Figure 2). All experiments were performed and repeated on at least three independent clones of each mutant with approximately equivalent levels of HA-STAT3.

To determine whether the levels of DN-STAT3 were sufficient to inhibit G-CSF-induced STAT3 activation, STAT3F, STAT3D, and vector control cells were serum- and growth factor-deprived and subsequently incubated with G-CSF. EMSAs with m67 oligonucleotides showed that stimulation of both nontransfected and vector control cells with G-CSF resulted in activation of a large amount of STAT3 homodimers as well as some STAT1-STAT3 heterodimers and STAT1 homodimers, as described previously (16). In contrast, G-CSF-induced STAT3 activation was greatly diminished in STAT3F and STAT3D cells (Figure 3). To study the effects of the DN-STAT3 mutants on STAT5 activation, EMSAs with β-casein oligonucleotides were performed. In cells that were serum- and growth factor-deprived before stimulation, G-CSF induced a strong but rapidly declining activation of STAT5 in vector control cells that was not influenced by DN-STAT3 (Figure 3). As expected, the formation of STAT3-STAT5 heteromeric complexes after prolonged G-CSF treatment was also abrogated by expression of DN-STAT3 (data not shown).

Dominant-negative STAT3 mutants prevent G-CSF-induced growth arrest. After switching from IL-3- to G-CSF-containing medium, both nontransfected and vector control cells proliferated in response to G-CSF for 5 to 7 days (Figure 4A). The cells then gradually stopped proliferating and developed into terminally differentiated neutrophils between days 8 and 11, showing the characteristic enlarged

cytoplasm-to-nucleus ratio, neutrophilic cytoplasm, segmented nuclei, and granules (Figure 4B). In contrast, cells overexpressing DN-STAT3 proliferated continuously in response to G-CSF. Both STAT3D and STAT3F expressing clones maintained an immature myeloblastic morphology and could be cultured in G-CSF-containing medium for at least 4 weeks (Figure 4). 32D transfectants overexpressing STAT3WT displayed growth and differentiation characteristics that were similar to nontransfected or vector control cells (Figure 4). This excludes that the effects of DN-STAT3 were merely caused by overexpression of STAT3 proteins, for instance by competing with other SH2-containing signaling proteins for docking to G-CSF-R.

We have previously established conditions in which addition of the cell cycle inhibitor cytosine arabinoside (Ara-C) results in accumulation of 32D cells in the G1 phase of the cell cycle, while leaving their ability to differentiate in response to G-CSF unaffected (24). In both IL-3- and G-CSF-containing medium, Ara-C-treated 32D cells arrest in G1, gradually lose viability and die after 4 to 5 days. Whereas cells cultured with IL-3 remain myeloblastic during this period, G-CSF induces terminal neutrophilic differentiation after 2 to 4 days, indicating that enforced cell cycle arrest does not induce differentiation of 32D cells in the absence of G-CSF (24). We applied these conditions to determine whether DN-STAT3 blocks induction of specific differentiation substrates or, instead, accumulation of cells in G1. In the presence of

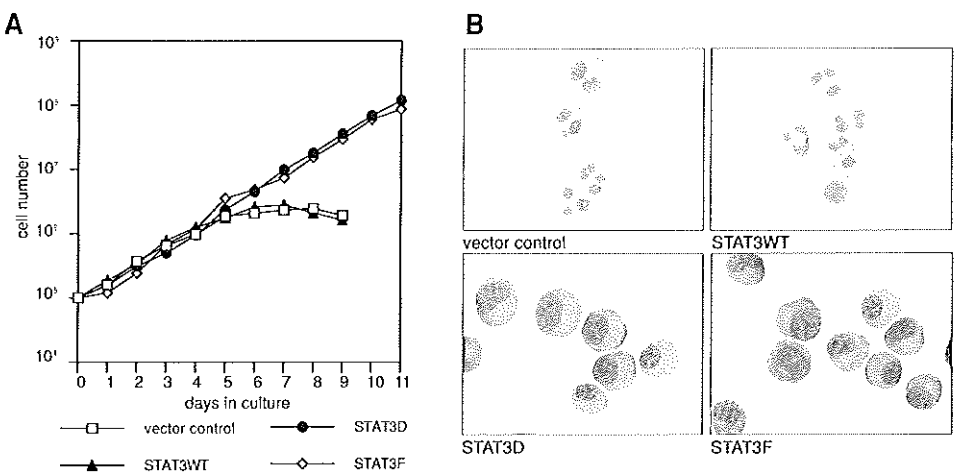

Figure 4. Effect of STAT3 variants on G-CSF responses of 32D cells. (A) Proliferation of 32D transfectants expressing STAT3 variants in response to G-CSF. The numbers of viable cells were determined on the basis of trypan blue exclusion at the indicated times. In the presence of IL-3, all clones proliferated with similar kinetics, whereas upon growth factor deprivation, cells died within 1 day without showing signs of neutrophilic differentiation. (B) Neutrophilic differentiation of 32D transfectants expressing STAT3 variants. Morphology of cells cultured for 9 days in the presence of G-CSF (May-Grünwald-Giemsa staining; original magnification: x 630).

TABLE 1. Neutrophilic differentiation of 32D cells expressing STAT3 variants

Cells	Neutrophilic differentiation[a]	
	G-CSF	G-CSF + Ara-C
vector control	63 ± 17	53 ± 21
STAT3WT	71 ± 14	38 ± 13
STAT3F	0	43 ± 11
STAT3D	0	34 ± 8

[a] Percentages of terminally differentiated neutrophils. Cells were cultured for 9 or 3 days in G-CSF-containing medium in the continuous presence or absence of Ara-C, respectively. At least 200 cells were scored. The means ± SE of 4 independent clones are shown.

Ara-C, G-CSF induced terminal neutrophilic differentiation in STAT3WT and vector control cells as well as in STAT3D and STAT3F cells with comparable efficiencies (Table 1). Thus, activation of STAT3 appears to be essential for G-CSF-mediated growth arrest, but not for induction of differentiation itself.

The $p27^{Kip1}$ promoter contains a functional STAT3 binding site. To obtain an indication as to how STAT3 mediates a growth arrest of 32D cells, we analyzed the available promoter sequences of cdk inhibitors. Previously, STAT responsive elements in the $p21^{WAF1/CIP1}$ promoter have been found that showed binding activity for STAT1 and STAT5 (29,32). We identified a potential STAT-binding site in the promoter of p27 at position -1585 (28). EMSAs with oligonucleotides derived from either the p21 or the p27 promoter were performed to verify whether G-CSF-activated STAT proteins indeed bind to these sequences. G-CSF induced the formation of three distinct nuclear complexes that bound to p27 oligonucleotides. Supershift analysis showed that the slowest migrating complex consists of STAT3 homodimers, the complex with intermediate mobility of STAT1-STAT3 heterodimers, and the fastest migrating complex of STAT1 homodimers (Figure 5A). EMSAs with p21 oligonucleotides on the same lysates indicated that G-CSF induced the formation of two distinct nuclear complexes with binding activity for the p21 promoter sequence (Figure 5B). Supershift analysis revealed that these complexes consist of STAT5 and STAT1 homodimers (data not shown). Because STAT1 homodimers are not activated and STAT5 homodimer formation is not increased upon G-CSF treatment compared with IL-3 stimulation (Figure 1), we do not consider this of functional importance for inducing a cell cycle arrest in these cells (see below). Overexpression of DN-STAT3 reduced the levels of STAT3 binding to p27 oligonucleotides, whereas binding of STAT5 to p21 oligonucleotides was unaffected (Figure 5), further supporting that STAT3 is involved in the regulation of p27, but not of p21, expression. Comparison

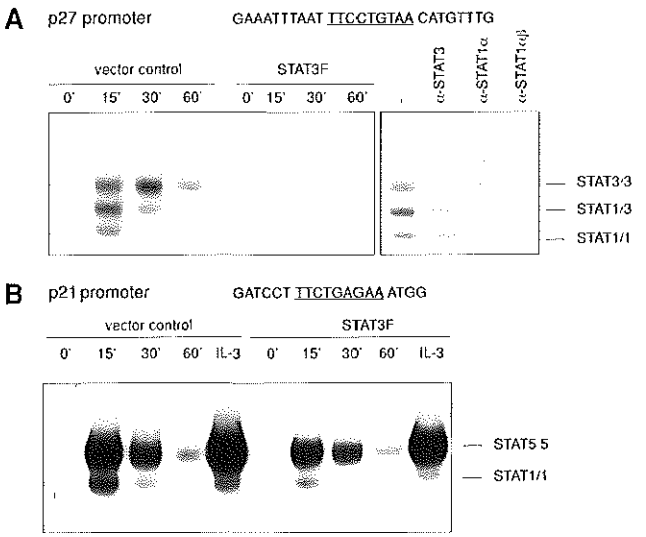

Figure 5. Identification of STAT proteins that bind to specific p27 or p21 promoter sequences by EMSA. Serum- and growth factor-deprived 32D transfectants were incubated at 37°C with G-CSF (100 ng/ml) for the times indicated or with IL-3 (100 ng/ml) for 15 minutes. Nuclear extracts were prepared and incubated with ^{32}P-labeled double-stranded p27 (A) or p21 (B) oligonucleotides. For supershift analysis, nuclear extracts from vector control cells stimulated for 15 minutes with G-CSF (100 ng/ml) were preincubated without (-) or with the indicated antibodies before the addition of ^{32}P-labeled p27 oligonucleotides.

of Figures 3 and 5 reveals a striking overlap in binding activity between p27 and m67 and between p21 and β-casein oligonucleotides. This was further confirmed by competition analyses. Binding of STATs to radioactive p27 oligonucleotides was efficiently competed by an excess of unlabeled m67, but not or hardly by p21 or β-casein probe. Conversely, an excess of β-casein, but not of p27 or m67 probe, prevented STAT binding to p21 oligonucleotides (data not shown). These observations are in agreement with the similarities in palindromic sequences between p27 (TTCCTGTAA) and m67 (TTCCCGTAA) and between p21 (TTCTGAGAA) and β-casein (TTCTAGGAA) oligonucleotides.

To investigate whether STAT3 directly induces *p27* gene expression, we performed reporter assays in HeLa cells transiently expressing a p27-promoter luciferase construct, a G-CSF-R expression plasmid, and different ratios of WT- and DN-STAT3. HeLa cells were used for these experiments because the differentiation competent 32D clone used in this study appeared to be unsuitable for transient transfections. In the presence of WT-STAT3, G-CSF induced a 7-fold increase in p27 promoter activity (Figure 6). The p27 promoter also contains a cAMP responsive element (CRE) at position -286 (28) and cAMP induces p27 expression in M-CSF-

treated macrophages (33) and in G-CSF-treated NFS-60 cells (34). As a control for the specific action of DN-STAT3 in this assay, we also treated cells with cholera toxin to elevate intracellular cAMP levels. p27 promoter activity was augmented approximately 2.4-fold by cholera toxin and 18-fold by G-CSF plus cholera toxin. As expected, increasing amounts of DN-STAT3 progressively inhibited transactivation induced by G-CSF, whereas CRE-driven luciferase activity was not affected (Figure 6).

STAT3 is essential for G-CSF-induced expression of $p27^{Kip1}$. To confirm the effect of STAT3 on p27 promoter activity, we investigated the ability of G-CSF to induce expression of p27 mRNA in 32D transfectants. Northern blot analyses showed that in both nontransfected and vector control cells p27 mRNA levels increased after 2 to 3 days of stimulation with G-CSF (Figure 7A). In contrast, p27 mRNA was not induced by G-CSF in 32D cells overexpressing DN-STAT3 (Figure 7A). We next examined whether increase in p27 mRNA results in induction of p27 protein expression during G-CSF-induced growth arrest and neutrophilic differentiation of 32D cells. In vector control cells, p27 protein appeared upon 3 days of stimulation with G-CSF, and reached a maximum after 5 to 7 days (Figure 7B). This timing of p27 protein expression is preceded by the increase in p27 mRNA and coincides with the observed growth arrest in these cells (Figure 4A). As expected, G-CSF did not induce p27 protein in 32D cells overexpressing DN-STAT3 (Figure 7B). During this prolonged G-CSF stimulation, STAT3 activation remained significantly diminished in

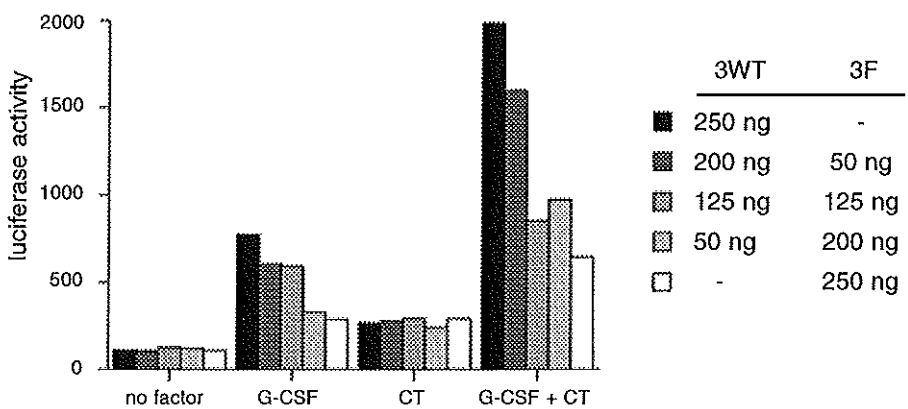

Figure 6. Effect of STAT3 variants on transactivation of p27 promoter in HeLa cells. HeLa cells were transfected with a p27-promoter luciferase reporter construct, a human G-CSF-R expression plasmid, and different amounts of constructs encoding STAT3WT or STAT3F as indicated. After transfection, cells were cultured for 48 hours without factor, with cholera toxin (200 ng/ml; CT), and/or with G-CSF (100 ng/ml). Cell lysates were prepared and assayed for luciferase activity. Luciferase light units of extracts from untransfected cells were used as basal level. Data represent the means of 4 independent experiments. Incubation with forskolin or dibutyryl-cAMP resulted in responses similar to those obtained with cholera toxin.

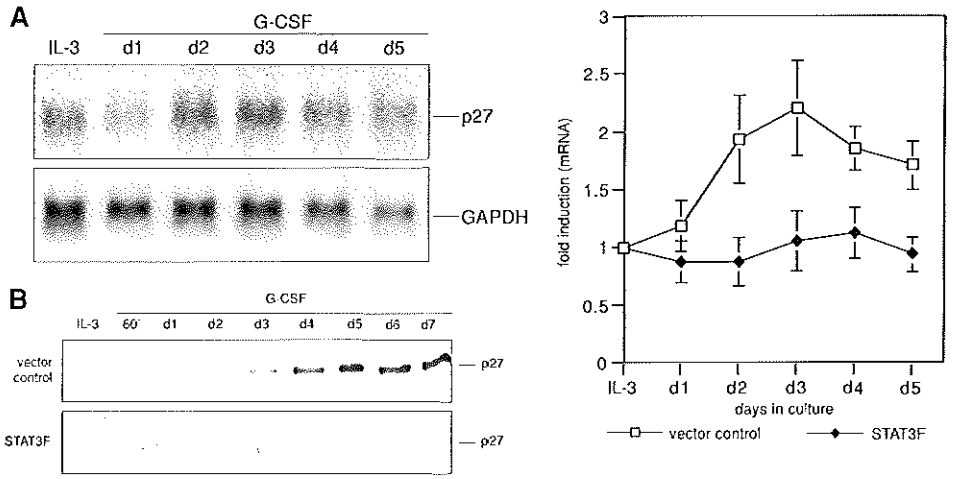

Figure 7. Induction of p27 expression in 32D transfectants. Cells were switched from IL-3- to G-CSF-containing medium after extensive washing to remove residual IL-3 and incubated at 37°C for the times indicated. (A) Total RNA (10 µg) of 32D/G-CSF-R cells was analyzed by Northern blot hybridization using a ^{32}P-labeled *p27* probe. The blot was reprobed with *GAPDH* to confirm equal loading. The graph shows quantitative analyses of *p27* mRNA induction, expressed as fold induction by G-CSF compared to by IL-3. The means ± SE of 3 independent clones are shown. (B) Lysates were prepared and analyzed by Western blotting with anti-p27 antibodies.

STAT3F and STAT3D cells as compared with vector control cells (data not shown). Reprobing of the blots with anti-p21 antibodies did not reveal any alterations in the (low) levels of expression of p21 protein (data not shown).

p27$^{-/-}$ mice show enhanced G-CSF-induced colony formation. To further confirm the involvement of p27 in the control of G-CSF-induced myeloid cell proliferation, we performed *in vitro* colony assays with bone marrow and spleen mononuclear cells of p27 knockout mice. The generation of p27-deficient mice has been previously described (31). All tissues of these mice are enlarged and contain more cells than of their wild-type littermates. Furthermore, the absence of p27 results in enhanced proliferation of hematopoietic progenitor cells in response to several growth factors. However, the effect of p27 deficiency on G-CSF-induced colony formation was not addressed. We therefore performed *in vitro* colony assays with cells of p27$^{-/-}$ and p27$^{+/+}$ mice in the presence of G-CSF, GM-CSF, or IL-3. In agreement with previous observations (31), the total number of GM-CSF-responsive progenitor cells per organ was increased in p27$^{-/-}$ mice compared with control littermates (femur: 1.5-fold; spleen: 2-fold; Figure 8A). Equivalent results were obtained for IL-3-responsive colony forming cells (femur: 1.4-fold; spleen: 2-fold; Figure 8A). Interestingly, the difference in G-CSF-responsive progenitors between

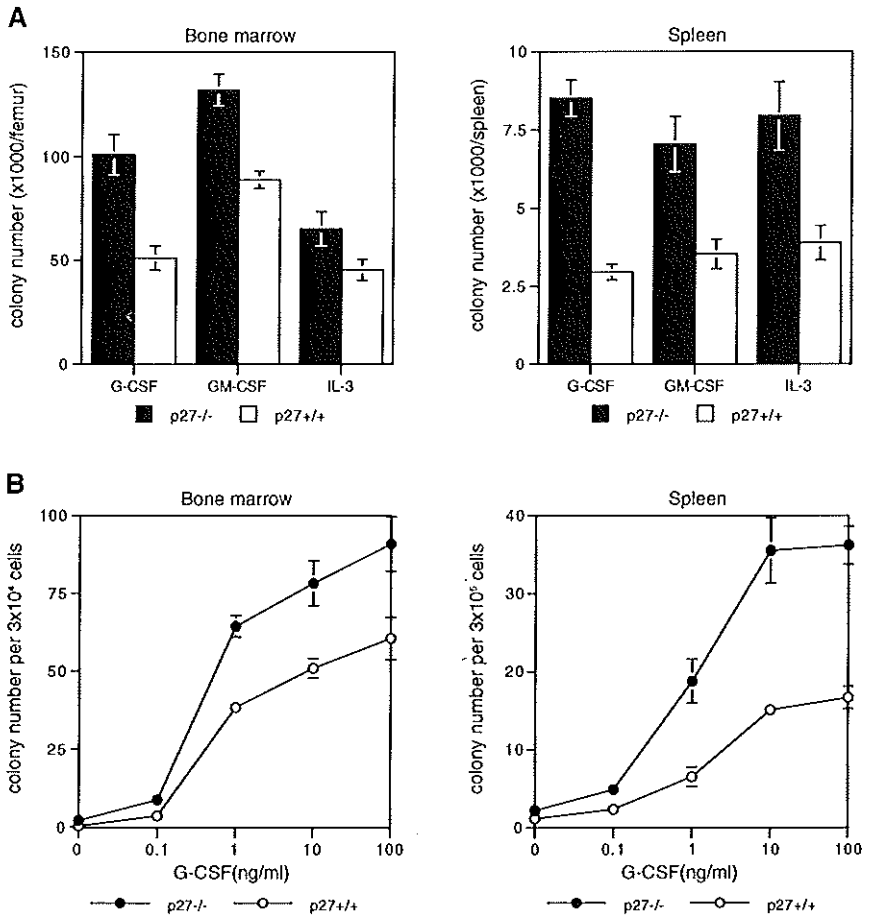

Figure 8. *In vitro* colony assays with bone marrow and spleen mononuclear cells of $p27^{-/-}$ and $p27^{+/+}$ mice. Cells were plated in methylcellulose-containing media supplemented with the indicated growth factors. Hematopoietic colonies containing 30 cells or more were scored after 7 days. Data represent the means ± SE of 3 male mice of each genotype. (A) Total numbers of colonies per organ are shown. (B) Numbers of G-CSF-responsive colony forming cells per 3 x 10^4 bone marrow or 3 x 10^5 spleen mononuclear cells are shown.

p27 knockout and wild-type mice was even more pronounced (femur: 2-fold; spleen: 2.9-fold; Figure 8A). These differences are partially due to the fact that both the bone marrow and the spleen of $p27^{-/-}$ mice contain approximately 1.3-fold more cells than of $p27^{+/+}$ mice. We therefore also determined the frequency of colony forming cells, i.e. the colony number per 3 x 10^4 bone marrow or 3 x 10^5 spleen mononuclear cells. Importantly, also the frequency of G-CSF-responsive colony forming cells was significantly increased in both the bone marrow (1.5-fold) and the spleen (2.2-fold) of

$p27^{-/-}$ mice compared to control littermates (Figure 8B). G-CSF concentrations required to reach maximal colony formation were similar. The increase in colony numbers might be the result of both a selective expansion of the G-CSF-responsive progenitor compartment in p27-deficient mice as well as of an enhanced proliferative capacity of the progenitor cells in response to G-CSF.

If p27 plays a key role in G-CSF-induced growth arrest that is a prerequisite for myeloid cells to proceed with differentiation, G-CSF-stimulated colonies derived from $p27^{-/-}$ mice are also expected to be larger in size and to contain less differentiated cells. Although the size of the colonies was similar on day 7 of culture, the average number of cells per colony on day 14 was twice as high in colonies derived from either bone marrow or spleen cells of $p27^{-/-}$ mice compared with those of $p27^{+/+}$ mice. Consistent with these findings, cytological examination of G-CSF-stimulated colonies on day 7 of culture demonstrated lower percentages of terminally differentiated neutrophils in colonies derived from $p27^{-/-}$ mice compared with $p27^{+/+}$ mice (i.e. 44 ± 6% vs. 67 ± 7% for bone marrow colonies, and 38 ± 4% vs. 52 ± 1% for spleen colonies). These results provide additional evidence supporting the hypothesis that p27 is involved in G-CSF-mediated neutrophilic differentiation by downmodulation of proliferation.

STAT3β does not affect G-CSF-induced proliferation and neutrophilic differentiation. The short form of STAT3, STAT3β, that differs from STAT3 by the replacement of the C-terminal 55 amino acid residues by 7 residues specific for STAT3β as a result of alternative splicing, is predominantly expressed in cells capable of differentiation (35). To examine a possible role of STAT3β in G-CSF-mediated responses, we first investigated whether the expression level of STAT3β is influenced by G-CSF during proliferation and neutrophilic differentiation of 32D cells. Although 32D cells express some STAT3β protein, G-CSF did not modulate the expression level, suggesting no direct involvement of STAT3β in the control of differentiation (Figure 9). To further substantiate this, we ectopically overexpressed STAT3β in G-CSF-R expressing 32D cells (Figure 10A). Overexpression of STAT3β did not interfere with G-CSF-induced STAT3 and STAT1 homo- and heterodimer

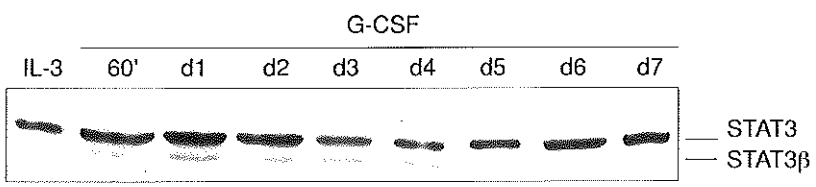

Figure 9. Expression of STAT3 isoforms in 32D/G-CSF-R cells. Cells were switched from IL-3- to G-CSF-containing medium after extensive washing to remove residual IL-3 and incubated at 37°C for the times indicated. Lysates were prepared and analyzed by Western blotting with anti-STAT3 antibodies (against amino acids 1-175).

formation (data not shown). STAT3β overexpressing cells did not show altered G-CSF-induced p27 protein expression and displayed growth and differentiation characteristics similar to vector control cells (Figures 10B and C). Furthermore, G-CSF-induced p27 promoter activity was not affected by transfection of STAT3β in HeLa cells, whereas the inhibition of transactivation by STAT3F could be fully restored by cotransfection of STAT3β (data not shown). Taken together, these data indicate that STAT3β does not act as a dominant-negative regulator of STAT3-mediated p27 induction.

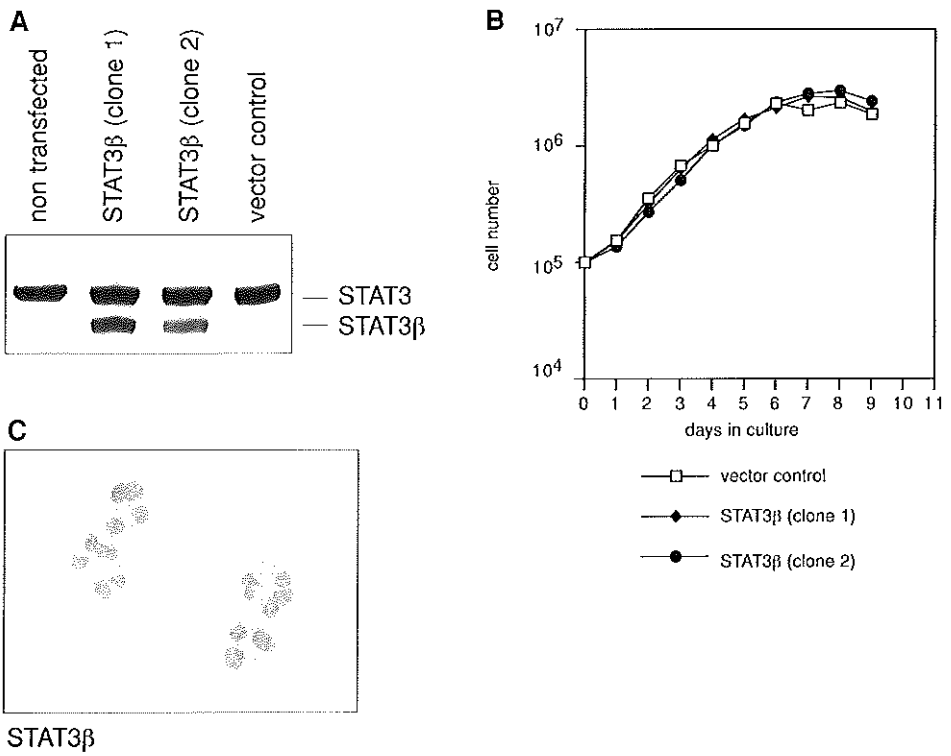

Figure 10. Overexpression of STAT3β in 32D/G-CSF-R cells. (A) Western blot analysis of lysates from 32D transfectants. The blot was hybridized with anti-STAT3 antibodies (against amino acids 1-175). (B) Proliferation of 32D transfectants in response to G-CSF. The numbers of viable cells were determined on the basis of trypan blue exclusion at the indicated times. (C) Neutrophilic differentiation of 32D transfectants overexpressing STAT3β. Morphology of cells cultured for 9 days in the presence of G-CSF (May-Grünwald-Giemsa staining; original magnification: x 630).

Discussion

STAT3 has recently been implicated in IL-6-induced monocytic and G-CSF-induced granulocytic differentiation (7,9), but which mechanisms are controlled by STAT3 remained unclear. Here, we have shown that the inhibitory effects of dominant-negative forms of STAT3 on G-CSF-induced neutrophilic differentiation of 32D cells could be overridden by the cell cycle inhibitor Ara-C, suggesting that STAT3 does not control differentiation itself, but induces a growth arrest that is essential for the cells to undergo differentiation.

Previously, STAT1 has been implicated in interferon-induced G1 arrest via the cdk inhibitor $p21^{WAF1/CIP1}$ (29). More recently, thrombopoietin-induced megakaryocytic differentiation and growth arrest was also functionally linked to expression of p21 (32). In both studies, potential STAT responsive elements in the p21 promoter were recognized with binding activity for STAT1 and STAT5, suggesting that p21 is a direct target for these STATs. In contrast, STAT3 did not bind to these elements, predicting that STAT3-mediated cell cycle arrest occurs via other mechanisms.

We have shown here that STAT3 controls cell cycle arrest by enhancing the expression of the p21 family member $p27^{Kip1}$, via direct binding to STAT3-binding elements in the p27 promoter region. Although thus far regulation of p27 expression has been mainly attributed to translational mechanisms (19-21), our data indicate that p27 is under the direct transcriptional control of STAT3. It is of note however that STAT3-DNA binding was already maximal 1 day after G-CSF induction, whereas p27 mRNA levels increased after 2 to 3 days and only after 5 to 7 days of G-CSF stimulation p27 protein was maximally expressed. There may be several explanations for this lag in p27 induction. Firstly, it is possible that additional mechanisms controlling STAT3-mediated transcription are involved, e.g. conformational changes of the STAT-containing complexes or interaction of the STAT complexes with other transcription regulating proteins. We have attempted to directly determine transcriptional activity of STAT3 during progressive differentiation of G-CSF-stimulated 32D cells, using a reporter construct containing four copies of the acute-phase response element in front of the minimal junB promoter linked to the *luciferase* gene. Unfortunately, we did not succeed in reproducible transfections of differentiating 32D cells, and therefore no conclusive data were obtained. Secondly, induction of the p27 promoter might require transcriptional synergy between STAT3 and other transcriptional regulators that are only induced after several days of G-CSF stimulation of 32D cells. The observation that neither dibutyryl-cAMP nor cAMP-inducing agents (cholera toxin, forskolin) significantly altered the kinetics of G-CSF-induced p27 expression in 32D cells (De Koning *et al.*, unpublished data) argues against a direct contribution of the cAMP responsive element (CRE) in the p27 promoter. In agreement with this, elevation of intracellular cAMP levels did not affect the proliferation and differentiation characteristics of 32D cells (De Koning *et al.*, unpublished data). A putative contribution of other candidate proteins with p27

promoter binding activity, e.g. Sp1, NFkB, and Myb, remains to be investigated (28). In view of this, it is of interest that Sp1 physically interacts and synergizes with STAT1 in IFN-γ-induced activation of the intercellular adhesion molecule-1 (ICAM-1) gene (36). Furthermore, induction of the CCAAT/enhancer binding protein δ promoter by IL-6 requires transcriptional synergy between Sp1 and STAT3 (37). Thirdly, p27 protein levels might remain low during the first 3 to 5 days of G-CSF stimulation due to ubiquitin-mediated degradation (38). Recent data indicate that mitogen-stimulated Ras activity induces signaling via two independent pathways that are both essential for cell cycle progression, namely a Ras/ERK pathway that induces cyclin D protein expression and a Ras/Rho pathway that results in ubiquitin-mediated p27 degradation (39). Importantly, Ras activation plays a crucial role in G-CSF-induced cell cycle progression of 32D cells (24,40). Thus, it is possible that during day 3 to 5 of G-CSF stimulation, when levels of activated Ras are high, low p27 protein levels are due to protein degradation despite active STAT3-mediated transcription.

The splice variant STAT3β is specifically expressed in hematopoietic cells capable of differentiation (35) and has some functional properties that are distinct from full-length STAT3. For instance, STAT3β, but not STAT3, acts synergistically with c-Jun in activating a promoter containing the IL-6-responsive element of the *α_2-macroglobulin* gene (41). It was therefore of interest to determine whether STAT3β is upregulated by G-CSF and could account for the delayed p27 expression. Expression levels of STAT3β did not increase during prolonged G-CSF stimulation of 32D cells. Furthermore, ectopic overexpression of STAT3β did not affect G-CSF responses of 32D cells. These data suggest that STAT3β is not involved in G-CSF-mediated p27 expression and also argue against a role of STAT3β as a dominant-negative regulator of STAT3-mediated transcription on the p27 promoter, as has been shown for the ICAM-1 promoter (25).

Previously, we detected point mutations in the *G-CSF-R* gene of patients with severe congenital neutropenia who showed disease progression to acute myeloid leukemia (42). These mutations introduce premature stop codons between amino acids 715 and 731 and result in the deletion of the C-terminal cytoplasmic region of the receptor that is essential for the induction of neutrophilic differentiation. Contrary to the transient proliferative signals provided by wild-type G-CSF-R, activation of G-CSF-R mutants lacking the C-terminal differentiation domain results in sustained and enhanced proliferation in myeloid cells. Such truncated G-CSF-R activate STAT3 in serum-and growth factor-deprived cells at lower levels (16,43). Tyrosine 704 of G-CSF-R mediates STAT3 activation in these G-CSF-R mutants, whereas wild-type G-CSF-R also activates STAT3 via the C-terminal region that is deleted in the truncated G-CSF-R. Whether the reduced levels of STAT3 activation induced by the G-CSF-R mutants are sufficient for p27 expression during prolonged G-CSF stimulation remains to be determined. This knowledge may provide important clues as to how G-CSF-mediated cell cycle control is affected in severe congenital

neutropenia by G-CSF-R mutations and how this could contribute to progression to acute myeloid leukemia.

After serum and growth factor deprivation of cells, G-CSF activates STAT1, STAT3, and STAT5 (14-16), but levels of activation return to base line 60 minutes after stimulation. The physiological significance of this early and transient peak of STAT activation is still unclear. Our present results show that, during progressive G-CSF-induced differentiation, only the induction of STAT3-containing complexes was robust and sustained. Importantly, besides STAT3 homodimers, also STAT3-STAT5 heteromers were formed, which were not detected during the early peak of STAT activation. The mobility of these STAT3-STAT5 complexes decreased after 3 days of culture, suggesting that prolonged G-CSF treatment resulted in additional conformational changes of these heteromeric complexes. Intriguingly, the appearance of these slower migrating STAT3-STAT5 heteromers correlated more tightly with the kinetics of p27 induction than the formation of STAT3 homodimers. Further study of the composition and function of the STAT3-STAT5 heteromeric complexes should provide insight into how these different STAT3-containing complexes contribute to control the balance between proliferation and differentiation of myeloid cells via upregulation of p27 protein.

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CHAPTER 7

General discussion

G-CSF-R transduces signals that control proliferation and survival of myeloid progenitor cells, and their differentiation towards neutrophilic granulocytes. Studies described in this thesis were performed to identify the signal transduction pathways activated by G-CSF-R that are coupled to proliferation and differentiation induction. In particular, the involvement of signals mediated by the cytoplasmic tyrosine residues of G-CSF-R was investigated, such as activation of the $p21^{Ras}$ pathway and STAT proteins. Tyrosine kinase activity induced by G-CSF results in the phosphorylation of the four conserved cytoplasmic tyrosines located in the membrane-distal region of G-CSF-R, providing binding sites for SH2 domain-containing signaling proteins. To determine the role of these tyrosines in G-CSF signaling, C-terminal deletion mutants and tyrosine-to-phenylalanine substitution mutants of G-CSF-R were constructed. The signal transduction abilities of the G-CSF-R mutants were tested in transfectants of the mouse pro-B cell line BAF3, and the myeloid cell line 32D. Involvement of the described signaling pathways in G-CSF-mediated cellular responses was investigated in a differentiation competent subclone of 32D cells that lacks endogenous G-CSF-R. In 32D cells transfected with wild-type *G-CSF-R* cDNA, G-CSF induces transient proliferation followed by terminal neutrophilic differentiation (Chapter 5). This is currently the most relevant cell line model available to study the effects of G-CSF-R mutants or signaling protein mutants on G-CSF-driven neutrophil development.

7.1 Mechanisms of $p21^{Ras}$ and STAT activation by G-CSF-R

In Chapter 2, it is shown that G-CSF stimulation of BAF3 cells expressing G-CSF-R proteins induces the formation of several signaling complexes of the $p21^{Ras}$ pathway, namely p145/Shc/GRB2, p90/GRB2, and SHP-2/GRB2 complexes (Figure 1). The C-terminal region of G-CSF-R containing the four tyrosine residues is essential for activation of these complexes. G-CSF-induced SHP-2/GRB2 association appeared to be partly mediated via Y704 and partly via other domain(s) of the C-terminal region of G-CSF-R. In contrast, Y764 of G-CSF-R is indispensable for the formation of p145/Shc/GRB2 and p90/GRB2 complexes. These observations were recently further substantiated in our laboratory by binding experiments with recombinant purified SH2 domains of several signaling proteins and with, on single tyrosines, phosphorylated mutant G-CSF-R proteins. Far Western blotting techniques were used to show that SHP-2 can directly bind to Y704 as well as to Y764, whereas both Shc and GRB2 can only bind directly to Y764 of G-CSF-R (1,2). All these findings suggest an important role for Y764 of G-CSF-R in G-CSF-mediated $p21^{Ras}$ signaling. This was confirmed by $p21^{Ras}$-loading assays showing that G-CSF-induced $p21^{Ras}$ activation is severely reduced by substitution of Y764 (Chapter 5).

The same BAF3 cell transfectants expressing the various G-CSF-R mutants were used to determine which cytoplasmic regions of G-CSF-R are involved in STAT activation. In Chapter 3, it is shown that the C-terminal region of G-CSF-R containing

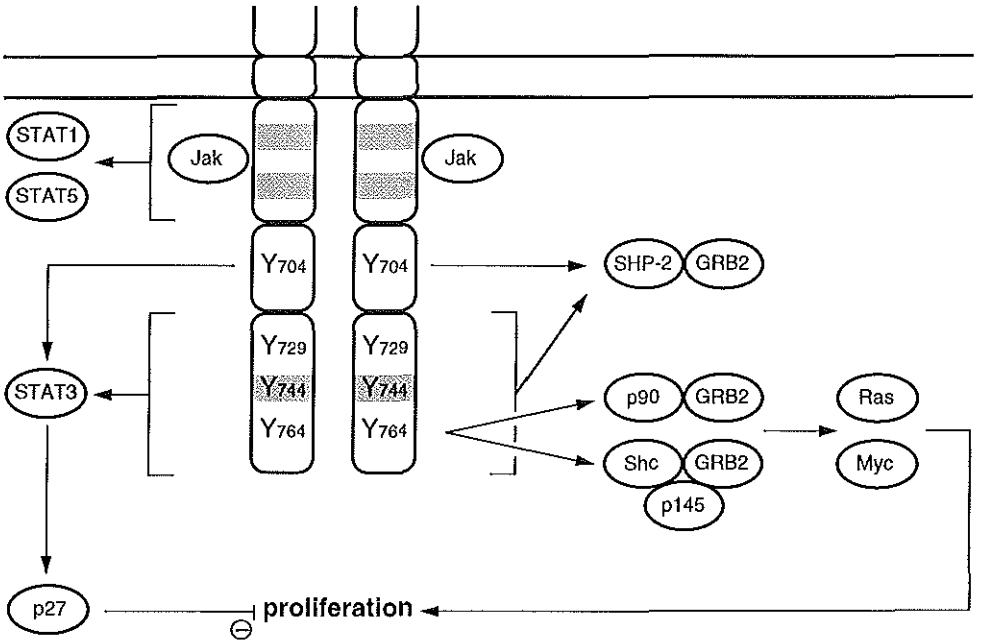

Figure 1. Signal transduction pathways activated by G-CSF-R. Data described in this thesis are summarized in a schematic diagram.

the cytoplasmic tyrosine residues is important for G-CSF-induced activation of STAT3 but not for STAT1 activation. Like the formation of SHP-2/GRB2 complexes, STAT3 activation is mediated via Y704 as well as via other domain(s) of the C-terminal region of G-CSF-R (Figure 1). To further study these alternative mechanisms of STAT3 activation, more detailed experiments were recently performed using G-CSF-R mutants in which all four tyrosine residues were substituted or which retain just one of the cytoplasmic tyrosines. In the BAF3 cell transfectants, the C-terminal region of G-CSF-R appeared to mediate STAT3 activation in a phosphotyrosine-dependent manner, via direct binding of STAT3 to either Y704 or Y744, and in a phosphotyrosine-independent manner, via as yet unknown interactions (3). Although not included in this thesis, we and others have also shown that G-CSF-induced activation of STAT5, like STAT1 activation, does not depend upon the C-terminal region and therefore does not require phosphotyrosine residues of G-CSF-R (4,5). Thus, the membrane-proximal region of G-CSF-R that is involved in Jak binding and activation is essential and sufficient for activation of STAT1 and STAT5 (Figure 1). In Chapter 3, the possibility is discussed that, in these cases, the Jak kinases may specifically recruit the STAT proteins. In support of this, it has recently been shown that the kinase-like JH2 domain of Jaks can interact with STAT proteins, resulting in direct STAT activation independent of receptor phosphorylation (6).

To further investigate the role of Jak kinases in G-CSF signaling, a G-CSF-R mutant was constructed in which tryptophan 650 in the membrane-proximal region was replaced by arginine (Chapter 4). This mutant was unable to activate Jak kinases, presumably due to interference with docking of Jaks to the receptor protein. In BAF3 cell transfectants, the tryptophan-to-arginine substitution completely abrogated the mitogenic response to G-CSF, as well as activation of STAT proteins and of signaling complexes of the $p21^{Ras}$ pathway. We hypothesized that Jak activation is an early event in G-CSF signaling that is essential for receptor phosphorylation (either directly or indirectly, i.e. via intermediate kinases) and thereby for the activation of signaling proteins that dock to these phosphotyrosines. This was confirmed by the observation that G-CSF-induced activation of STAT3 was blocked by pretreatment of BAF3 cells with the specific Jak inhibitor AG-490 (3,7). In contrast, preliminary experiments indicate that pretreatment of 32D cells with this Jak inhibitor only abrogates G-CSF-induced STAT5 activation, whereas STAT3 activation is unaffected. To further substantiate this, experiments are in progress to determine the signaling ability of the G-CSF-R tryptophan-to-arginine mutant in 32D cells. Furthermore, STAT3 activation in 32D cells appeared to be mediated by G-CSF-R in a phosphotyrosine-dependent manner only, thus via interaction with either Y704 or Y744. These discrepancies between 32D and BAF3 cells suggest that G-CSF-R phosphorylation and STAT3 activation in 32D cells are independent of Jak activity, i.e. mediated via other tyrosine kinases. In agreement with this, recent studies have provided evidence that the tyrosine kinases Src, Fes, and Bmx can all mediate STAT phosphorylation without the involvement of Jak kinases (8-10). Which tyrosine kinases are involved in G-CSF-induced receptor phosphorylation and/or STAT3 activation in 32D cells remains to be established. One obvious candidate is the Src family kinase Lyn, which is constitutively associated with the G-CSF-R complex in a phosphotyrosine-independent manner and is activated by G-CSF (11). By using G-CSF-R expressing transfectants of $Lyn^{-/-}$ chicken B cell lines, it was shown that G-CSF-induced proliferation and Shc phosphorylation are mediated by Lyn independently of Jak kinases (12,13). Moreover, studies with Jak1-deficient and Jak2-deficient mice indicate that neither Jak1 nor Jak2 alone are essential for G-CSF responses (14,15). Although the Jak kinases activated by G-CSF-R may fulfill redundant functions, it is conceivable that other cytoplasmic kinases are involved in G-CSF-R signaling as is suggested above.

7.2 Control of proliferation by G-CSF-R

In Chapter 6, the involvement of STAT3 in G-CSF-induced proliferation and neutrophilic differentiation was examined by overexpressing dominant-negative STAT3 mutants in 32D cells expressing wild-type G-CSF-R. STAT3 appeared to be essential for G-CSF-mediated neutrophilic differentiation by inducing a growth arrest that is a prerequisite for myeloid precursor cells to proceed with differentiation.

Evidence is provided that STAT3 induces cell cycle exit via upregulation of the cdk inhibitor $p27^{Kip1}$ (Figure 1). Since disruption of the *STAT3* gene results in very early embryonic lethality due to unknown deficiencies, these observations could not be confirmed in STAT3-deficient cells (16). Besides the effects of STAT3 on proliferation, several recent studies have suggested involvement of STAT3 in survival signaling (17-19). Interestingly, preliminary experiments with 32D cells expressing G-CSF-R mutants in which all four tyrosine residues were substituted or which retain just one of the cytoplasmic tyrosines show a clear correlation between the ability of these mutants to activate STAT3 and to prevent apoptosis (Ward *et al.*, unpublished data). Interference of dominant-negative STAT3 mutants with G-CSF-induced survival signals is currently under investigation.

Whereas STAT3 seems to have a negative effect on mitogenesis, STAT5 activation has been implicated in proliferation induction. For instance, it was shown that expression of a constitutively active STAT5 mutant in IL-3-dependent BAF3 cells is capable of inducing cytokine-independent growth (20). Conversely, a dominant-negative STAT5 protein partially inhibited IL-3-induced proliferation of BAF3 cells (21). Furthermore, STAT5A/B-deficient mice were recently generated that showed a reduced response of bone marrow cells to IL-3 as well as to G-CSF in *in vitro* colony assays, suggesting involvement of STAT5 in IL-3- and G-CSF-mediated cell growth (22). Whether STAT5 is important for G-CSF-induced proliferation of 32D cells is currently examined by transfection of constitutively active or dominant-negative STAT5 mutants.

In Chapter 5, the role of the cytoplasmic tyrosine residues of G-CSF-R in the transduction of proliferation and differentiation signals was determined by expressing tyrosine-to-phenylalanine substitution mutants in 32D cells that lack endogenous G-CSF-R. All tyrosines could be replaced essentially without affecting the neutrophilic differentiation signaling properties of G-CSF-R. However, substitution of one specific tyrosine, i.e. Y764, markedly influenced proliferation induction, whereas substitution of Y704, Y729, or Y744 had no effect. G-CSF-induced cell cycle progression from the G1 to the S phase as well as activation of Shc and $p21^{Ras}$ and the induction of *c-myc* expression were abrogated by mutation of Y764. The potential involvement of these signaling events in proliferation induction is extensively discussed in Chapter 5. Additional evidence supporting the observation that Y764 of G-CSF-R is important for proliferation signaling is provided by recent experiments with a triple tyrosine-to-phenylalanine substitution mutant which retains only Y764 (mD), and a quadruple 'null' mutant with no cytoplasmic tyrosines (mO). Strikingly, mutant mO appeared incapable of transducing proliferation signals in 32D cell transfectants, whereas 32D cells expressing mutant mD proliferated continuously in response to G-CSF (1). Besides confirming the importance of Y764 for proliferation induction, these results also suggest that proliferation inhibitory signals are given via (one of) the other cytoplasmic tyrosines of G-CSF-R. This becomes clear by comparing the transient with the sustained proliferation from wild-type G-CSF-R and mutant mD, respectively. Because STAT3 activation is mediated via interaction with

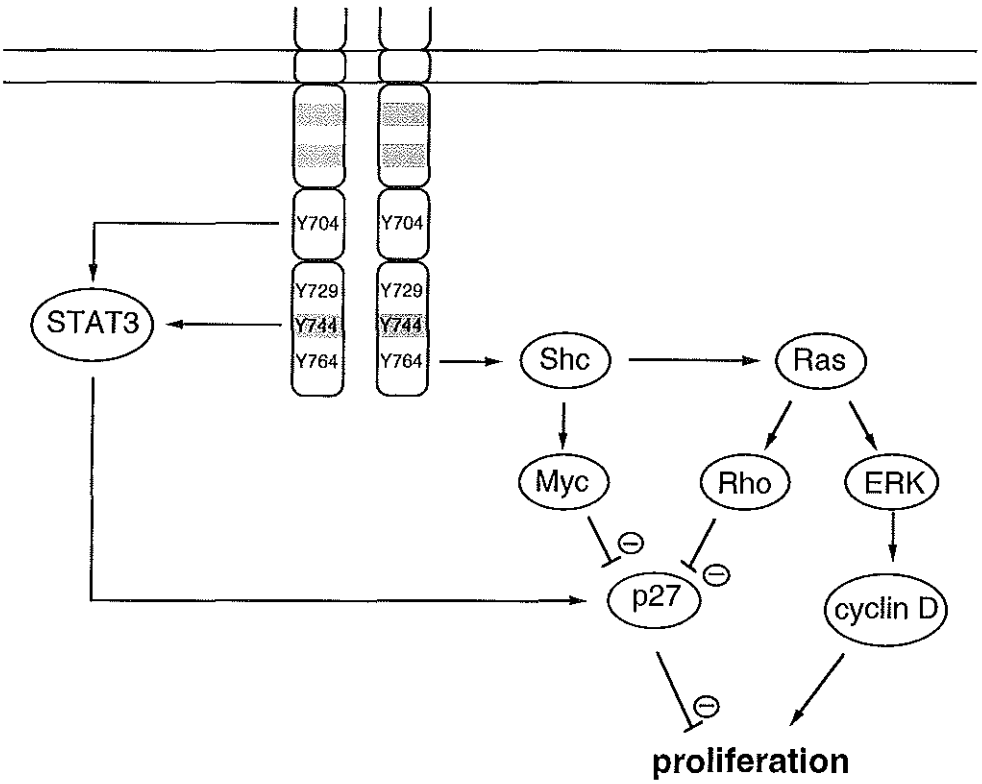

Figure 2. A model for G-CSF-R-controlled myeloid proliferation. STAT3, which is activated via Y704 or Y744 of G-CSF-R, inhibits proliferation of myeloid cells via upregulation of $p27^{Kip1}$. In contrast, Y764 of G-CSF-R is essential for proliferation induction and for activation of Shc, $p21^{Ras}$ and Myc. Via an ERK-dependent pathway, $p21^{Ras}$ induces the expression of cyclin D, and, via a Rho-dependent pathway, it stimulates degradation of $p27^{Kip1}$. Both mechanisms positively influence cell cycle entry. Myc probably affects cell growth via multiple pathways, one of which involves inhibition of $p27^{Kip1}$ function and thereby stimulates proliferation.

either Y704 or Y744 of G-CSF-R, a plausible proliferation inhibitory mechanism is STAT3-mediated induction of $p27^{Kip1}$ protein expression (Chapter 6). Alternatively, other signaling mechanisms might also be involved in downmodulation of proliferation during prolonged G-CSF stimulation. For instance, $p21^{Ras}$ activation plays a crucial role in G-CSF-induced cell cycle progression of 32D cells (Chapter 5) (23). However, it is unknown whether $p21^{Ras}$ activation by G-CSF is sustained. Recent data indicate that mitogen-stimulated $p21^{Ras}$ activity induces signaling via two independent pathways that are both essential for cell cycle progression, namely a $p21^{Ras}$/ERK pathway that induces cyclin D protein expression and a $p21^{Ras}$/Rho

pathway that results in ubiquitin-mediated $p27^{Kip1}$ degradation (Figure 2) (24). During prolonged G-CSF stimulation of myeloid cells, $p21^{Ras}$ activation might be inhibited, possibly resulting in decreased cyclin D expression and decreased $p27^{Kip1}$ degradation, and thereby in growth arrest. One possible mechanism of $p21^{Ras}$ inhibition is via induction of GTPase activating protein (GAP) family members. For instance, the neurofibromatosis type 1 (*NF1*) tumor-suppressor gene encodes a protein (neurofibromin) that accelerates GTP hydrolysis on $p21^{Ras}$ proteins. Interestingly, expression of neurofibromin is induced during myoblast differentiation (25). Furthermore, loss of NF1 function results in increased $p21^{Ras}$ activation and leads to aberrant growth of myeloid cells (26,27). However, no evidence was obtained so far that GAP proteins are recruited by the cytoplasmic tyrosines of G-CSF-R.

In addition to $p21^{Ras}$, Myc has been implicated in the control of myeloid cell proliferation (discussed in Chapter 5). Myc is a transcription factor, which must dimerize with Max proteins in order to regulate the activation of genes involved in cell proliferation. One product of an as yet unknown Myc/Max target gene(s) has been suggested to induce sequestration of $p27^{Kip1}$ in a form unable to bind cyclin/cdk complexes (28). In this way, Myc could counteract growth arrest by $p27^{Kip1}$ without $p27^{Kip1}$ degradation, which represents an essential aspect of Myc's mitogenic functions (Figure 2). During cellular differentiation, a reduction of Myc protein and a concomitant increase in Mad or Mxi proteins is frequently observed (29,30). Mad and Mxi proteins also form heterodimers with Max proteins and subsequently bind to similar sites as Myc/Max dimers. However, Mad/Max and Mxi/Max dimers repress transcription by ternary complex formation with the repressor Sin3, and thereby inhibit proliferation (31). During growth arrest of 32D cells after prolonged G-CSF stimulation, a significant increase of Mad1 expression was detected, whereas Myc expression only slightly decreased (Soede-Bobok *et al.*, unpublished data). Increased Mad expression may result in the repression of Myc/Max target genes, one of which encodes the as yet unidentified protein involved in sequestration of $p27^{Kip1}$ away from cyclin/cdk complexes. Besides the observed G-CSF-induced increase in $p27^{Kip1}$ protein expression, also more $p27^{Kip1}$ protein can then bind to, and thereby inhibit, cyclin/cdk complexes. This may provide an additional mechanism of G-CSF-mediated growth control. In agreement with this, granulocytic precursors of Mad1-deficient mice show an increased proliferative response to G-CSF at a late stage of neutrophil development (32).

7.3 Differentiation signaling by G-CSF-R

Although significant progress has been made in understanding G-CSF-R-controlled myeloid cell proliferation, it is still unclear which signal transduction pathways are involved in G-CSF-induced neutrophilic differentiation. In Chapter 5, it was shown that mutation of Y704, Y729, Y744, or Y764 did not affect the ability of G-CSF-R to transduce differentiation signals in 32D cells. Furthermore, G-CSF also

induces terminal neutrophilic differentiation in 32D cells expressing a triple mutant which retains only Y704 (mA), indicating that differentiation signaling by the C-terminal domain of G-CSF-R is tyrosine-independent (33). Additional experiments will be required to determine the signaling substrates that are recruited by this domain and initiate the differentiation process.

Because differentiation-specific signal transduction pathways have not yet been identified, the role of HGFs in the regulation of cellular differentiation remains controversial. Two general models for hematopoietic differentiation have been proposed (34,35). In the stochastic model, the ability to differentiate is an intrinsic feature of hematopoietic cells that is independent of HGFs. According to this model, HGFs only provide growth and survival signals that are required for the execution of the differentiation programs. In the instructive model, differentiation is actively induced by HGFs. The observation that the C-terminal domain of G-CSF-R specifically transduces neutrophilic differentiation signals in several myeloid cell line models (32D, L-GM, and FDC-P1 cells) provides evidence for the latter model (36,37). Notably, the same G-CSF-R domain is not functional in the pro-B cell line BAF3, suggesting that signaling for neutrophilic differentiation by G-CSF-R requires the appropriate intracellular environment or genetic program of the responding cells (36).

Recently, it was shown that truncation of the C-terminal differentiation domain of G-CSF-R causes neutropenia in a 'knock-in' mouse model, providing *in vivo* evidence that the G-CSF-R C-terminus is essential for normal neutrophil production (38). However, bone marrow cells from these mice differentiated *in vitro* to morphologically mature neutrophils in response to G-CSF. Similarly, G-CSF-R-deficient mice have chronic neutropenia, but do not show accumulation of immature granulocytic cells in the bone marrow, suggesting normal differentiation of the residual granulocytic precursors (39). These results indicate that G-CSF-R is a major regulator of granulopoiesis, although, at least in mice, a G-CSF-R-independent mechanism of neutrophil development also appears to exist. This conclusion is still compatible with the cell line data and the instructive model, since alternative granulocytic differentiation signals (e.g. from other HGFs) that are absent in the cell line models may compensate for the loss of G-CSF-R signals in primary cells.

Additional evidence supporting the instructive model of differentiation is presented in Chapter 5. In the presence of the cell cycle inhibitor cytosine arabinoside (Ara-C), 32D cells expressing wild-type G-CSF-R arrest in G1, gradually lose viability, and die after 4 to 5 days. Whereas cells cultured with IL-3 remain myeloblastic during this period, G-CSF induces terminal neutrophilic differentiation after 2 to 4 days, indicating that enforced cell cycle arrest does not induce differentiation of 32D cells in the absence of G-CSF. In agreement with this, suppression of apoptosis by overexpression of bcl-2 or bcl-x_L is not sufficient to support myeloid differentiation of 32D cells (40). Furthermore, Ara-C-treated 32D cells expressing a G-CSF-R mutant lacking the C-terminal differentiation-domain remain myeloblastic in both IL-3- and G-CSF-containing medium (De Koning *et al.*,

unpublished data). Taken together, these results suggest that neutrophilic differentiation requires both downmodulation of proliferation and, via the C-terminal cytoplasmic region of G-CSF-R, induction of specific differentiation signals (Figure 3).

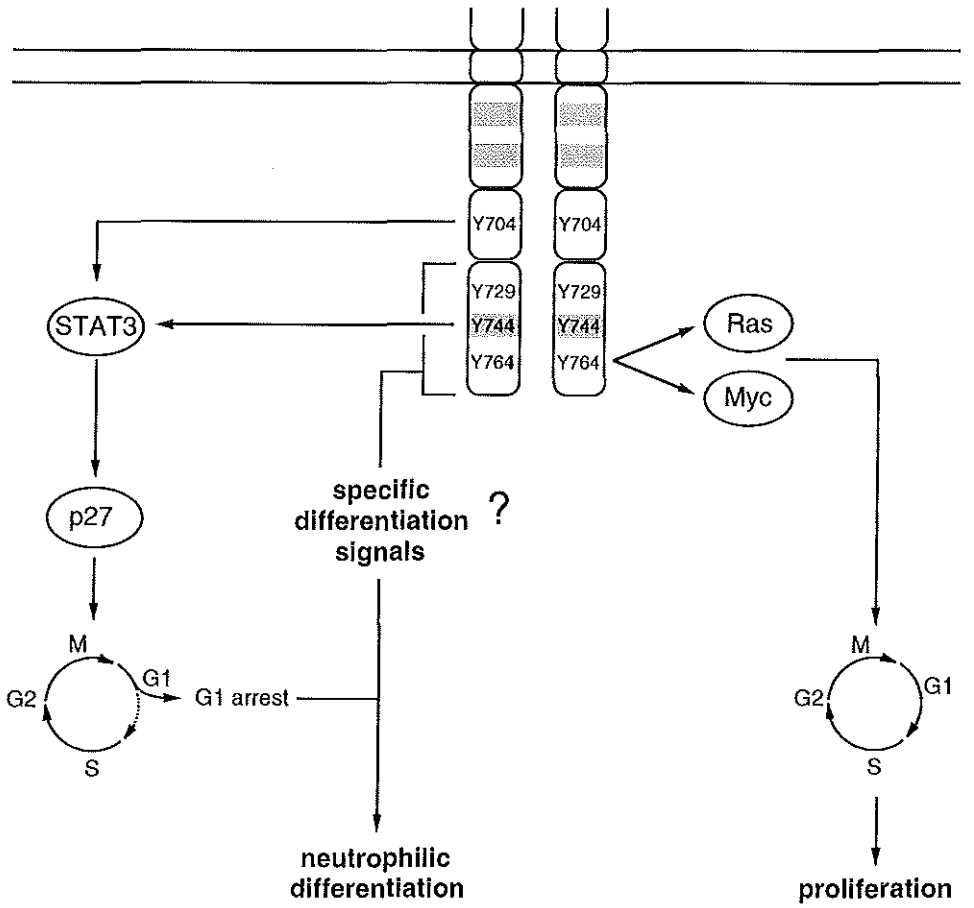

Figure 3. A model for the balance between G-CSF-R-controlled proliferation and differentiation induction during neutrophil development. Signaling via Y764 of G-CSF-R, which involves $p21^{Ras}$ and Myc activation, is essential for G-CSF-induced proliferation. In contrast, STAT3 inhibits proliferation of myeloid cells via upregulation of $p27^{Kipl}$. Besides accumulation of cells in the G1 phase of the cell cycle which is a prerequisite for differentiation, specific differentiation signals induced via the C-terminal cytoplasmic region of G-CSF-R are required for terminal neutrophilic differentiation.

7.4 Function of mutant G-CSF-R in severe congenital neutropenia

In 15 to 20% of patients with severe congenital neutropenia (SCN), mutations are found in the *G-CSF-R* gene (41,42). These mutations introduce premature stop codons resulting in the truncation of 82 to 98 C-terminal amino acids (Figure 4). SCN patients who develop AML almost invariably acquired a G-CSF-R mutation, suggesting that this genetic alteration represents a key step in leukemogenesis. Upon ectopic expression in myeloid cell lines, the truncated G-CSF-R consistently fails to transduce differentiation signals, whereas proliferation signaling by these receptors is sustained and enhanced. Moreover, the truncated receptors interfere with signaling induced via wild-type G-CSF-R in a dominant-negative manner (41). This dominant hyperproliferative function of truncated G-CSF-R was recently confirmed in a 'knock-in' mouse model with an equivalent G-CSF-R mutation (38). Daily treatment of these mice with G-CSF results in an increased production of neutrophils, leading to a sustained neutrophilia. This indicates that the G-CSF-R mutation itself is sufficient for a hyperproliferative response to G-CSF, supporting the idea that G-CSF-R truncation represents a preleukemic event.

To identify the mechanisms mediating the dominant hyperproliferative function of the truncated G-CSF-R, receptor activation and internalization studies were performed with both primary cells and 32D cell transfectants (33,43). G-CSF-induced

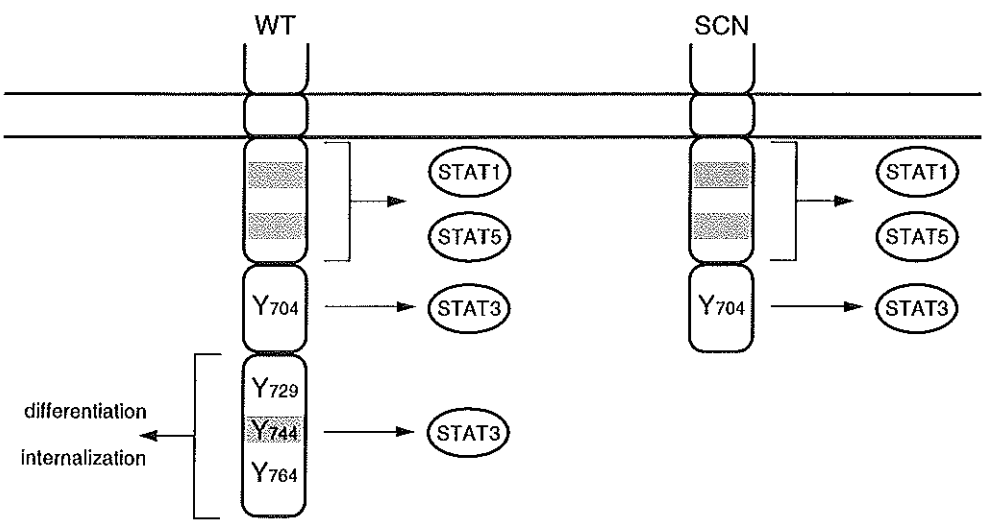

Figure 4. Schematic diagram of truncated G-CSF-R from SCN patients. Nonsense mutations in the *G-CSF-R* gene of SCN patients result in truncations of the C-terminal cytoplasmic region. The domains of wild-type (WT) and truncated G-CSF-R involved in induction of differentiation, internalization, and STAT activation are indicated.

internalization of truncated G-CSF-R appeared to be severely impaired which results in prolonged receptor activation. Importantly, mutant receptors were found to act in a dominant-negative manner over wild-type receptors with regard to both receptor activation and internalization. Currently, experiments are in progress to determine the signaling mechanisms activated via the C-terminal domain of G-CSF-R that mediate receptor internalization. These findings suggest that extended signaling by truncated G-CSF-R due to defective internalization results in hyperproliferation. In support of this, activation of STAT5, implicated in proliferation induction, is significantly prolonged due to receptor truncation (33,43). In contrast, the duration of STAT3 activation is hardly affected, because wild-type G-CSF-R already induces sustained activation of STAT3 (Chapter 6). However, the level of STAT3 activation by truncated G-CSF-R is clearly reduced compared to wild-type G-CSF-R. Most likely, this is due to the fact that the truncated G-CSF-R can only activate STAT3 via Y704, whereas STAT3 recruitment by wild-type G-CSF-R can also occur via Y744 of G-CSF-R (Figure 4). Whether the reduced levels of STAT3 activation by the G-CSF-R mutants prevent induction of $p27^{Kip1}$ protein expression during prolonged G-CSF stimulation remains to be determined. Although the increased STAT5 and the decreased STAT3 activation may already result in enhanced proliferation, it is obvious that sustained activation of other signaling pathways induced by the truncated G-CSF-R may also contribute to the observed hyperproliferation.

In summary, these results implicate G-CSF-R truncations as possible preleukemic events. It is conceivable that cells with an acquired G-CSF-R mutation may have a growth advantage due to prolonged receptor activation in the presence of ligand.

7.5 Future directions

Although it is still unclear which signal transduction pathways that are activated by G-CSF-R mediate neutrophilic differentiation, recent studies have suggested that several transcription factors are important for myeloid differentiation. Evidence supporting the involvement of transcription factors in myelopoiesis include the observations that many genes cloned at the site of leukemic translocation breakpoints are transcription factors, and that knockout mice of these factors show severe defects in myeloid development. The transcription factors shown to be important for induction of myeloid-specific genes are PU.1 (Spi-1), C/EBPα, C/EBPε, AML1, CBF, c-Myb, RARα, PLZF, and MZF1 (44). Their activity is modulated by co-activator and co-repressor proteins that create a physical bridge between transcription factors and the basal transcriptional machinery. Whether transcription factors either activate or repress transcription might be regulated by their ability to associate with histone acetyltransferases (co-activators; e.g. p300/CBP, pCAF) or histone acetylases (co-repressors; e.g. N-CoR, mSin3), respectively. Furthermore, it has recently been shown that the activity of a single transcription factor, Pit-1, can be modulated by

distinct signal transduction pathways, not through modification of Pit-1 itself, but through regulation of the recruited co-activator complex (45). How transcription factors are induced during myeloid differentiation and how they fit into G-CSF-R signaling cascades remains an important goal for future research.

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SUMMARY

&

SAMENVATTING (Summary in Dutch)

Summary

G-CSF is the most important growth factor involved in granulopoiesis and maintenance of neutrophil levels in the peripheral blood. It stimulates proliferation and survival of myeloid progenitor cells, and their differentiation towards neutrophilic granulocytes. G-CSF exerts its function via activation of a membrane receptor, G-CSF-R. In *Chapter 1*, an overview is given of the current understanding of the function of G-CSF-R in normal granulopoiesis as well as in some patients with severe congenital neutropenia and acute myeloblastic leukemia, diseases characterized by disturbed myeloid differentiation. The cytoplasmic domain of G-CSF-R contains discrete functional regions, suggesting the existence of multiple signaling pathways that are activated via these regions to elicit distinct biological effects. The C-terminal region of the human G-CSF-R contains four conserved tyrosine (Y) residues, located at positions 704, 729, 744, and 764, that could be involved in G-CSF-R-mediated signal transduction via interaction with SH2 domain-containing signaling proteins.

In *Chapter 2*, the ability of wild-type, C-terminal deletion mutants, and tyrosine-to-phenylalanine substitution mutants of G-CSF-R to activate signaling intermediates of the $p21^{Ras}$ pathway was examined. Several different Shc- and/or GRB2-containing complexes were formed after activation of G-CSF-R, namely p145/Shc/GRB2, p90/GRB2, and SHP-2/GRB2 complexes. Neither of these complexes was detected after activation of a C-terminal deletion mutant of G-CSF-R that lacks all four conserved cytoplasmic tyrosine residues. G-CSF induced the formation of SHP-2/GRB2 complexes in all the tyrosine-substitution mutants, suggesting that this association did not depend on the presence of single specific tyrosine residues in G-CSF-R. In contrast, Y764 of G-CSF-R appeared to be indispensable for the formation of p145/Shc/GRB2 and p90/GRB2 complexes, suggesting a prominent role for Y764 of G-CSF-R in G-CSF signal transduction.

In *Chapter 3*, the cytoplasmic regions of G-CSF-R involved in activation of STAT1 and STAT3 were determined. Studies with C-terminal deletion mutants and tyrosine-to-phenylalanine substitution mutants of G-CSF-R indicated that Y704 of G-CSF-R, located within a YXXQ consensus sequence for STAT3-SH2 binding, is involved in the recruitment and activation of STAT3 by G-CSF-R. However, STAT3 binding is not exclusively mediated by Y704, but also by other domains of the C-terminal region of G-CSF-R via as yet unknown interactions. In contrast, G-CSF-induced activation of STAT1 did not depend upon the membrane-distal cytoplasmic region of G-CSF-R, and therefore does not require phosphotyrosine residues of the receptor.

In *Chapter 4*, the role of Jak kinases in activation of the $p21^{Ras}$ pathway was investigated by constructing a mutant in which tryptophan 650 in the membrane-proximal region of G-CSF-R was replaced by arginine. This replacement abolished Jak phosphorylation and abrogated the mitogenic response to G-CSF. Significantly, the ability of G-CSF-R to induce formation of p145/Shc/GRB2, p90/GRB2, and

SHP-2/GRB2 complexes was also completely blocked as a result of this amino acid substitution. These data indicate that the membrane-proximal cytoplasmic region of G-CSF-R is not only crucial for proliferative signaling and activation of Jak kinases, but is also required for activation of signaling complexes of the $p21^{Ras}$ pathway, which occurs via the membrane-distal region of G-CSF-R.

In *Chapter 5*, the role of the cytoplasmic tyrosine residues of G-CSF-R in transduction of proliferation and differentiation signals was determined by expressing tyrosine-to-phenylalanine substitution mutants in a differentiation competent subclone of 32D cells that lacks endogenous G-CSF-R. All tyrosines could be replaced essentially without affecting the neutrophilic differentiation signaling properties of G-CSF-R. However, substitution of one specific tyrosine, i.e. Y764, markedly influenced proliferation induction as well as the timing of differentiation, whereas substitution of Y704, Y729, or Y744 had no effect on proliferation signaling. Mutant Y764F failed to support G-CSF-induced cell cycle progression from the G1 to the S phase, resulting in accelerated differentiation and significantly reduced net production of mature neutrophils. Importantly, G-CSF-mediated activation of Shc and $p21^{Ras}$ and the induction of *c-myc* expression were severely reduced by substitution of Y764. These findings indicate that Y764 of G-CSF-R is crucial for maintaining the proliferation/differentiation balance during G-CSF-driven neutrophil development, and suggest a role for multiple signaling mechanisms in maintaining this balance.

In *Chapter 6*, the involvement of STAT3 in G-CSF-induced proliferation and neutrophilic differentiation was examined by overexpressing dominant-negative STAT3 mutants in differentiation competent 32D cells expressing the wild-type G-CSF-R. STAT3 appeared to be essential for G-CSF-induced neutrophilic differentiation by inducing a growth arrest that is a prerequisite for myeloid precursor cells to proceed with differentiation. The splice variant STAT3β, previously shown to be a dominant-negative form of STAT3 on certain promoters, did not affect cell growth. To obtain an indication as to how STAT3 mediates a growth arrest of 32D cells, the role of several cyclin-dependent kinase (cdk) inhibitors was analyzed. The level of the cdk inhibitor $p27^{Kip1}$ appeared to increase during G-CSF stimulation of 32D cells. An oligonucleotide derived from the promoter region of p27 containing a putative STAT-binding site shifted with STAT3 in electrophoretic mobility shift assays. Dominant-negative STAT3 reduced G-CSF-induced p27 promoter activity in luciferase reporter assays and interfered with the expression of both p27 mRNA and protein. Furthermore, bone marrow and spleen mononuclear cells of p27-deficient mice showed and increased proliferative capacity in response to G-CSF. These results suggest that STAT3-controlled cell cycle exit of myeloid precursors is mediated via direct upregulation of the cdk inhibitor p27.

In *Chapter 7*, the findings described in this thesis are summarized and their significance for the understanding of the function of G-CSF-R is discussed.

Samenvatting (Summary in Dutch)

De productie van neutrofiele granulocyten (granulopoiese) wordt gereguleerd door een netwerk van hematopoietische groeifactoren. Eén van die groeifactoren, G-CSF, speelt een essentiële rol in dit proces. G-CSF stimuleert de proliferatie en overleving van de granulocytaire voorlopercellen en induceert hun differentiatie tot neutrofiele granulocyten. Deze verschillende effecten worden geïnitieerd na binding van G-CSF aan specifieke receptor eiwitten (G-CSF-R) op het celoppervlak, wat resulteert in activatie van intracellulaire signaal transductie routes. In *Hoofdstuk 1* wordt een overzicht gegeven van de huidige inzichten in de functie van G-CSF-R in zowel normale granulopoiese als bij patiënten met ernstige aangeboren neutropenie en acute myeloide leukemie. In het cytoplasmatische domein van G-CSF-R kunnen meerdere subdomeinen met specifieke functies worden herkend. Dit suggereert dat door deze subdomeinen verschillende signaalwegen worden geactiveerd om de afzonderlijke biologische effecten te induceren. Het C-terminale deel van de humane G-CSF-R bevat 4 geconserveerde tyrosine residuen (Y704, Y729, Y744 en Y764), die betrokken zouden kunnen zijn bij G-CSF-R gemedieerde signaal transductie door interacties aan te gaan met SH2 domeinen van signaal eiwitten.

In *Hoofdstuk 2* wordt beschreven welke eiwitten van de $p21^{Ras}$ route na G-CSF stimulatie worden geactiveerd. G-CSF-R activatie blijkt de vorming van verschillende Shc en/of GRB2 bevattende complexen (p145/Shc/GRB2, p90/GRB2 en SHP-2/GRB2) te bewerkstelligen. Deze complexen worden niet gevormd na activatie van een C-terminale deletie mutant van G-CSF-R, waarin de 4 cytoplasmatische tyrosines ontbreken. Met behulp van tyrosine-naar-phenylalanine substitutie mutanten is vervolgens aangetoond dat G-CSF geïnduceerde SHP-2/GRB2 associatie niet afhankelijk is van één specifieke tyrosine van G-CSF-R. Daarentegen blijkt Y764 essentieel te zijn voor de vorming van zowel p145/Shc/GRB2 als p90/GRB2 complexen. Dit suggereert een belangrijke rol voor Y764 van G-CSF-R in G-CSF geïnduceerde signaal transductie.

In *Hoofdstuk 3* wordt uiteengezet welke cytoplasmatische subdomeinen van G-CSF-R een rol spelen in de activatie van STAT1 en STAT3. Y704 van G-CSF-R is gelegen in een zogenaamde YXXQ consensus sequentie, waarvan bekend is dat het een interactie aan kan gaan met het SH2 domein van STAT3. Uit experimenten met C-terminale deletie en tyrosine-naar-phenylalanine substitutie mutanten van G-CSF-R blijkt dat Y704 inderdaad betrokken is bij activatie van STAT3. Het C-terminale deel van G-CSF-R bevat naast Y704 ook andere domeinen die, via nog onbekende mechanismen, STAT3 activatie bewerkstelligen. G-CSF afhankelijke activatie van STAT1 verloopt echter niet via het membraan-distale cytoplasmatische domein van G-CSF-R en is dus onafhankelijk van de tyrosine residuen van de receptor.

In *Hoofdstuk 4* is de betrokkenheid van Jak kinases bij de activatie van de $p21^{Ras}$ route onderzocht door een G-CSF-R mutant te maken, waarin tryptofaan residu 650 in het membraan-proximale domein van G-CSF-R vervangen is door een arginine

residu. Deze substitutie blijkt zowel G-CSF-R gemedieerde tyrosine-fosforylering van Jak2 als proliferatie te verhinderen. Tevens is deze mutant niet in staat om de vorming van p145/Shc/GRB2, p90/GRB2 en SHP-2/GRB2 complexen te induceren. Deze resultaten tonen aan dat het membraan-proximale cytoplasmatische domein van G-CSF-R niet alleen essentieel is voor proliferatie inductie en activatie van Jak kinases, maar ook voor activatie van signaal eiwitten van de $p21^{Ras}$ route, wat via het membraan-distale domein van G-CSF-R verloopt.

In *Hoofdstuk 5* is de rol van de cytoplasmatische tyrosine residuen van G-CSF-R in de transductie van proliferatie en differentiatie signalen onderzocht door tyrosine-naar-phenylalanine substitutie mutanten van G-CSF-R te expresseren in myeloide 32D cellen. Afzonderlijke mutatie van de 4 tyrosines blijkt geen invloed te hebben op de differentiatie-inducerende eigenschappen van G-CSF-R. Daarentegen heeft de substitutie van één specifieke tyrosine, nl. Y764, een dramatisch effect op zowel de inductie van proliferatie als de timing van differentiatie. Mutatie van Y704, Y729 of Y744 beïnvloedt de proliferatie signalering niet. G-CSF stimulatie van de mutant van Y764 induceert geen celcyclus progressie van de G1 naar de S fase, wat resulteert in versnelde differentiatie en een significant verlaagde productie van neutrofiele granulocyten. Daarnaast heeft substitutie van Y764 een sterk remmend effect op de G-CSF gemedieerde activatie van Shc and $p21^{Ras}$ en de inductie van *c-myc* expressie. Deze bevindingen tonen aan dat Y764 van G-CSF-R essentieel is voor een uitgebalanceerde proliferatie/differentiatie inductie tijdens G-CSF gereguleerde productie van neutrofiele granulocyten en suggereren dat meerdere signaal transductie routes hierbij betrokken zijn.

In *Hoofdstuk 6* wordt beschreven welke rol STAT3 speelt in G-CSF afhankelijke proliferatie en differentiatie inductie. Dit is onderzocht door dominant-negatieve STAT3 mutanten te overexpresseren in 32D cellen getransfecteerd met de wild-type G-CSF-R. STAT3 blijkt essentieel te zijn voor G-CSF gemedieerde differentiatie door de proliferatie te remmen, wat een voorwaarde is voor granulocytaire voorlopercellen om te differentiëren. Alhoewel de splice variant STAT3β de activiteit van STAT3 op sommige promoters dominant-negatief beïnvloedt, blijkt STAT3β geen effect te hebben op de proliferatie van 32D cellen. Om een aanwijzing te krijgen hoe STAT3 de proliferatie van 32D cellen reguleert, is de betrokkenheid van cyclin-dependent kinase (cdk) remmers onderzocht. De expressie van de cdk remmer $p27^{Kip1}$ wordt verhoogd tijdens G-CSF stimulatie van 32D cellen. Vervolgens is met EMSA aangetoond, dat een oligonucleotide, afgeleid van een sequentie die in de promoter regio van p27 voorkomt, in staat is om STAT3 te binden. Dominant-negatief STAT3 verlaagt de G-CSF geïnduceerde p27 promoter activiteit in luciferase reporter experimenten en verhindert de expressie van zowel p27 mRNA als p27 eiwit. Verder blijkt dat de G-CSF gemedieerde proliferatie van granulocytaire voorlopercellen afkomstig uit beenmerg en milt van p27-deficiente muizen verhoogd is in vergelijking met wild-type muizen. Deze resultaten suggereren dat STAT3 de proliferatie van granulocytaire voorlopercellen reguleert door inductie van p27 expressie.

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Curriculum vitae

De schrijver van dit proefschrift werd op 4 december 1968 te Rotterdam geboren. Na het behalen van zijn VWO diploma aan de Scholengemeenschap 'Blaise Pascal' te Spijkenisse in 1987 studeerde hij één jaar Biologie aan de Universiteit Utrecht, waarvan het propedeutisch examen werd gehaald. Na herplaatsing stapte hij in 1988 over naar de studie Medische Biologie aan de Universiteit Utrecht. In augustus 1993 haalde hij zijn doctoraal examen (*cum laude*) met als hoofdvak Medische Microbiologie en als bijvakken Moleculaire Biologie en Immunologie. Tijdens de doctoraalstudie deed hij onderzoek naar *Staphylococcus epidermidis* infecties bij premature neonaten o.l.v. Prof. Dr. A. Fleer (Microbiologie, Wilhelmina Kinderziekenhuis, Utrecht) en Prof. Dr. H. van Dijk (Medische Microbiologie, Academisch Ziekenhuis Utrecht) en naar de relatie tussen structuur en functie van interleukine-3 o.l.v. Dr. Ir. L.C.J. Dorssers (Moleculaire Biologie, Dr. Daniël den Hoed Kliniek, Rotterdam) en Dr. Ir. G.T. Rijkers (Immunologie, Wilhelmina Kinderziekenhuis, Utrecht). In september 1993 begon hij aan het in dit proefschrift beschreven onderzoek bij de afdeling Hematologie van de Dr. Daniël den Hoed Kliniek en het Instituut Hematologie van de Erasmus Universiteit Rotterdam o.l.v. Dr. I.P. Touw en Prof. Dr. B. Löwenberg. Na zijn promotie zal hij als research fellow in dienst van het 'Koningin Wilhelmina Fonds' onderzoek gaan verrichten naar polygene kanker predispositie. Hiermee zal worden begonnen op het UCSF Cancer Center te San Francisco o.l.v. Dr. A. Balmain (Onyx Pharmaceuticals), waarna het onderzoek wordt voortgezet bij de vakgroep Fysiologische Chemie van de Universiteit Utrecht o.l.v. Prof. Dr. J.L. Bos.