

Novel Point Mutation in the Extracellular Domain of the Granulocyte Colony-stimulating Factor (G-CSF) Receptor in a Case of Severe Congenital Neutropenia Hyporesponsive to G-CSF Treatment

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Summary

Severe congenital neutropenia (SCN) is a heterogeneous condition characterized by a drastic reduction in circulating neutrophils and a maturation arrest of myeloid progenitor cells in the bone marrow. Usually this condition can be successfully treated with granulocyte colony-stimulating factor (G-CSF). Here we describe the identification of a novel point mutation in the extracellular domain of the G-CSF receptor (G-CSF-R) in an SCN patient who failed to respond to G-CSF treatment. When this mutant G-CSF-R was expressed in myeloid cells, it was defective in both proliferation and survival signaling. This correlated with diminished activation of the receptor complex as determined by signal transducer and activator of transcription (STAT) activation, although activation of STAT5 was more affected than STAT3. Interestingly, the mutant receptor showed normal affinity for ligand, but a reduced number of ligand binding sites compared with the wild-type receptor. This suggests that the mutation in the extracellular domain affects ligand-receptor complex formation with severe consequences for intracellular signal transduction. Together these data add to our understanding of the mechanisms of cytokine receptor signaling, emphasize the role of *GCSFR* mutations in the etiology of SCN, and implicate such mutations in G-CSF hyporesponsiveness.

Key words: cytokine • receptor • signal transduction • cell survival • structure activity relationship

Severe congenital neutropenia (SCN)¹ is a heterogeneous disease state characterized by a severe reduction in circulating neutrophils ($<0.2 \times 10^9/\text{liter}$), and a maturation arrest of bone marrow progenitor cells at the promyelocyte/myeloid stage (1–3). Myeloid progenitor cells from SCN patients frequently show reduced responsiveness to G-CSF in vitro (4, 5). Moreover, treatment with pharmacological doses of G-CSF is able to restore the neutrophil count in the majority of SCN patients (5–7).

G-CSF is known to play a crucial role in the regulation of granulopoiesis by stimulating the proliferation, survival, and maturation of myeloid progenitor cells (8–11). The

various biological effects of G-CSF are mediated through the G-CSF receptor (G-CSF-R), a member of the hematopoietin receptor superfamily which forms homooligomeric complexes upon ligand binding (12, 13). The G-CSF-R, like other hematopoietin receptors, lacks intrinsic tyrosine kinase activity but activates cytoplasmic tyrosine kinases (9, 12). Signal transduction pathways that involve Janus tyrosine kinases (Jak1, Jak2, and Tyk2) and signal transducer and activator of transcription proteins (STATs 1, 3, and 5) have been linked to the G-CSF-R (14–22). Activation of STAT3 has been implicated in G-CSF-mediated differentiation (23), whereas STAT5 appears to have an important function in G-CSF-mediated proliferation and survival (24).

We have previously identified in a subset of SCN patients nonsense mutations in the gene encoding the G-CSF-R that truncate the COOH-terminal domain of the receptor

¹Abbreviations used in this paper: CRH, cytokine receptor homologous; RT, reverse transcription; SCN, severe congenital neutropenia; STAT, signal transducer and activator of transcription.

(25–27). When expressed in myeloid cells, these truncated receptors transduce a strong growth signal but are defective in maturation signaling (26, 28). This is due to sustained receptor activation caused by defective internalization and an altered balance of STAT activation (22, 29, 30). However, in SCN patients with this mutation, G-CSF treatment is able to reverse the neutropenia (25–27).

Here we describe the analysis of an SCN patient unresponsive to standard G-CSF treatment. We identify a novel point mutation in the extracellular domain of the G-CSF-R in this patient. This mutation changes a highly conserved proline to histidine at position 206 of the cytokine receptor homologous (CRH) domain, which is part of a proline-rich “hinge” motif located between the NH₂- and COOH-terminal “barrels” of the CRH domain. When this mutant receptor was expressed in myeloid 32D cells, it was defective in both G-CSF-mediated proliferation and survival. This correlated with greatly diminished activation of the receptor complex, and severe but selective abrogation of signaling from the mutant G-CSF-R. The mutant receptor showed a normal affinity of ligand binding, but a reduction in the number of ligand binding sites per receptor. This suggests that the mutation in the extracellular domain alters the architecture of the ligand-receptor complex, which severely affects intracellular signaling and cellular responses to G-CSF. These data contribute to our understanding of cytokine receptor signaling, and further emphasize the role of *GCSFR* mutations in the etiology of both SCN and hyporesponsiveness to G-CSF therapy.

Materials and Methods

Patient Details. The patient is female, born November 1990, the only child of healthy, unrelated parents. At birth, the patient's absolute neutrophil count was 0×10^9 /liter. During the first 18 mo of life, the patient experienced four episodes of cellulitis and abscesses. From June 1992 to October 1993, the patient was treated with a series of escalating daily subcutaneous doses of G-CSF from 10 to 70 μ g/kg, with no response; peripheral polymorphonuclear cells remained at 0×10^9 /liter, and bone marrow smears revealed a maturation arrest at the myelocyte stage, indicative of severe congenital neutropenia. In January 1994, a continuous intravenous 24-h infusion of 150–200 μ g/kg G-CSF was administered to the patient for 10 d consecutively with no response and persistent marrow maturation arrest. The patient continued to have recurrent bacterial infections.

Reverse Transcription PCR and Nucleotide Sequence Analysis. Total RNA was isolated from bone marrow mononuclear cells using acid guanidinium isothiocyanate extraction (31), and reverse-transcribed using oligo(dT) primers and MMLV reverse transcriptase (GIBCO BRL). PCR amplification was performed using the primer pairs GRFR3 (5'-TCGGAAAGGTGAAG-TAACTTGTC) and GRRV5 (5'-TCCATGGGATCAAGACACAG) to amplify nucleotides 106–832, and GRFR5 (5'-TG-CAGGCAGAGAATGCGCTG) and GRRV4 (5'-AGGGTCCATGGTGTCCCTGGTACA) to amplify nucleotides 772–1707 of the *GCSFR* cDNA. The products were cloned into the TA cloning vector (Invitrogen) and sequenced using the dideoxy chain termination method with T7 polymerase (Amersham Pharmacia Biotech).

Analysis of Genomic DNA. The *GCSFR* mutation identified in patient AR introduces a new BsiHkAI site (G^A/tGC^A/tC) (see Fig. 1 B). Therefore, to analyze for the presence of the mutation, genomic DNA was prepared from fibroblast and blood cells using standard techniques, and subjected to PCR with the primer pair GRFR16 (5'-ACCTAGAGAGAAACAAAGAC) and GRRV15 (5'-AGCGGATGCAGCGTATCT). The resultant products were digested with BsiHkAI (New England Biolabs) before separation on a 3% agarose gel.

Cell Culture. The subline of the IL-3-dependent murine myeloid cell line 32Dcl3, called 32D.cl8.6 (29), and the IL-3-dependent murine pro-B cell line Ba/F3 (32) were maintained in RPMI 1640 medium supplemented with 10% FCS and 10 ng/ml of murine IL-3, at 37°C and 5% CO₂.

Plasmid Construction, Transfections, and Infections. Cloning of the wild-type (WT) *GCSFR* cDNA into the eukaryotic expression vector pLNCX (33) has been described previously (25). To clone the mutant receptor (mAR) into this vector, a multistep procedure was required. First, the region spanning the extracellular region of the mutant receptor was obtained by joining the 5' and 3' regions obtained using reverse transcription (RT)-PCR via a common BsmI site at position 782, to produce plasmid pTAR1600. From this construct, the region encoding the mutation was obtained as an *Ava*I fragment which was used to replace the equivalent region of the WT *GCSFR* cloned into pBlueScript. The entire receptor coding region was then subcloned into pLNCX as a *Hind*III-*Cla*I fragment. The pLNCX expression constructs were linearized by *Pvu*I digestion and transfected into 32D.cl8.6 or Ba/F3 cells by electroporation. After 48 or 24 h of incubation, respectively, cells were selected with G418 (GIBCO BRL) at a concentration of 0.8 or 1.2 mg/ml, respectively, with multiple clones expanded for further analysis. For retroviral infections, the WT and mAR *GCSFR* cDNAs were cloned into pBabe, as described (25). The resulting plasmids were transfected into the Phoenix A amphotropic packaging cell line (Clontech) to generate recombinant retrovirus, following standard protocols. Virus supernatants were used to infect 32D cells harboring the pLNCX.GCSFR(WT) vector (32D[WT^{Neo}]) using RetroNectinTM (Takara Biomedicals), as described by the manufacturer. After 48 h, puromycin was added to 1 μ g/ml to select for stably transduced cells.

Analysis of G-CSF-R Expression. To determine G-CSF-R expression levels, cells were incubated at 4°C for 60 min sequentially with 10 μ g/ml of biotinylated mouse anti-human G-CSF-R mAb LMM741 (PharMingen), 5 μ g/ml of PE-conjugated streptavidin, 5 μ g/ml of biotinylated antistreptavidin antibody, and finally 2 μ g/ml of PE-conjugated streptavidin, with washing between each antibody step. Samples were analyzed by flow cytometry using a FACScanTM (Becton Dickinson). Several independently derived cell lines of each construct were selected on the basis of homogeneous receptor expression. The *GCSFR* mutation identified in patient AR introduced a new BsiHkAI site (G^A/tGC^A/tC). Therefore, as confirmation of mutant receptor expression, RT-PCR was performed with primers GRFR5 and GRRV11 (5'-ACTGTCCTGGGGTCCAGCTG), and the resultant products were cut with BsiHkAI before electrophoresis on an agarose gel.

Analysis of G-CSF Binding. For routine analysis of G-CSF binding, biotinylated G-CSF protein was used, followed by 5 μ g/ml of PE-conjugated streptavidin, as described (28). To estimate the affinities of the G-CSF binding sites, G-CSF binding experiments and Scatchard analysis were performed using [¹²⁵I]-G-CSF (800–1,500 Ci/mmol; Amersham Pharmacia Biotech), as described (34).

DNA Synthesis Assay. DNA synthesis was assessed by [^3H]-thymidine (^3H -TdR) incorporation. Cells (5×10^4) 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 24 h. 16 h before cell harvest, 0.1 μCi of ^3H -TdR (2 Ci/mmol; Amersham Pharmacia Biotech) was added to each well. ^3H -TdR incorporation was measured by harvesting cells on glass fiber filters followed by counting on a Packard Top Count scintillation counter.

Cell Proliferation and Morphological Analysis. To determine proliferation, cells were incubated at an initial density of $1\text{--}2 \times 10^5$ cells/ml in 10% FCS/RPMI medium supplemented with 100 ng/ml of human G-CSF, 10 ng/ml of murine IL-3, or without growth factors. The medium was replenished every 1–2 d, and the cell densities were adjusted to $1\text{--}2 \times 10^5$ 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.

Preparation of Nuclear Extracts. Cells were deprived of serum and factors for 4 h at 37°C in RPMI 1640 medium at a density of 10^6 /ml, and then stimulated with either RPMI 1640 medium alone or in the presence of 100 ng/ml human G-CSF. At different time points, 10 vol of ice-cold PBS supplemented with 10 μM Na_3VO_4 were added. Subsequently, cells were pelleted and resuspended in ice-cold hypotonic buffer (20 mM Hepes, pH 7.8, 20 mM NaF, 1 mM Na_3VO_4 , 1 mM $\text{Na}_4\text{P}_2\text{O}_7$, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.2% Tween-20, 0.125 μM okadaic acid, 1 mM Pefabloc SC, 50 $\mu\text{g}/\text{ml}$ aprotinin, 50 $\mu\text{g}/\text{ml}$ leupeptin, 50 $\mu\text{g}/\text{ml}$ bacitracin, and 50 $\mu\text{g}/\text{ml}$ iodoacetamide) (35). Cells were vortexed for 10 s, and the nuclei were pelleted by centrifugation at 15,000 g for 30 s. Nuclear extracts were prepared by resuspension of the nuclei in high-salt buffer (hypotonic buffer with 420 mM NaCl and 20% glycerol) and extraction of proteins by rocking for 30 min at 4°C . Insoluble materials were removed by centrifugation at 4°C for 15 min at 15,000 g .

Electrophoretic Mobility Shift Assay. Nuclear extracts of $\sim 0.5 \times 10^6$ cells were incubated for 20 min at room temperature with 0.2 ng of ^{32}P -labeled double-stranded oligonucleotide ($5\text{--}10 \times 10^3$ 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, and 8% glycerol) (36). The oligonucleotide probes used in this study were m67 (5'-CATTCCCGTAAATC), a high-affinity mutant of the sis-inducible element (SIE) of the human *c-fos* gene (37), which binds STAT1 and STAT3, and β -cas (5'-AGATTTCTAGGAATTCAATCC), derived from the 5' region of the β -casein gene (38), which binds STAT5 and STAT1. The DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gels containing 5% glycerol in $0.25 \times$ TBE. The gels were dried and subsequently analyzed by autoradiography.

Results

Identification of a Mutation in the G-CSF-R Extracellular Domain in an SCN Patient Hyporesponsive to G-CSF Therapy. Patient AR represents an unusual SCN patient, being totally unresponsive to standard and high-dose G-CSF therapy. Subsequently, the patient responded to combined prednisone therapy in combination with G-CSF, the details of which will be published elsewhere (Ward, A.C., I.P. Touw, and M.H. Freedman, manuscript in preparation). We had previously reported that no mutations were present in the sequence encoding the cytoplasmic domain of the G-CSF-R of this patient (27). However, the unusual phenotype of this patient prompted us to analyze the complete sequence of the *GCSFR*. This identified a C \rightarrow A mutation at nucleotide 850 of the *GCSFR* cDNA, which pro-

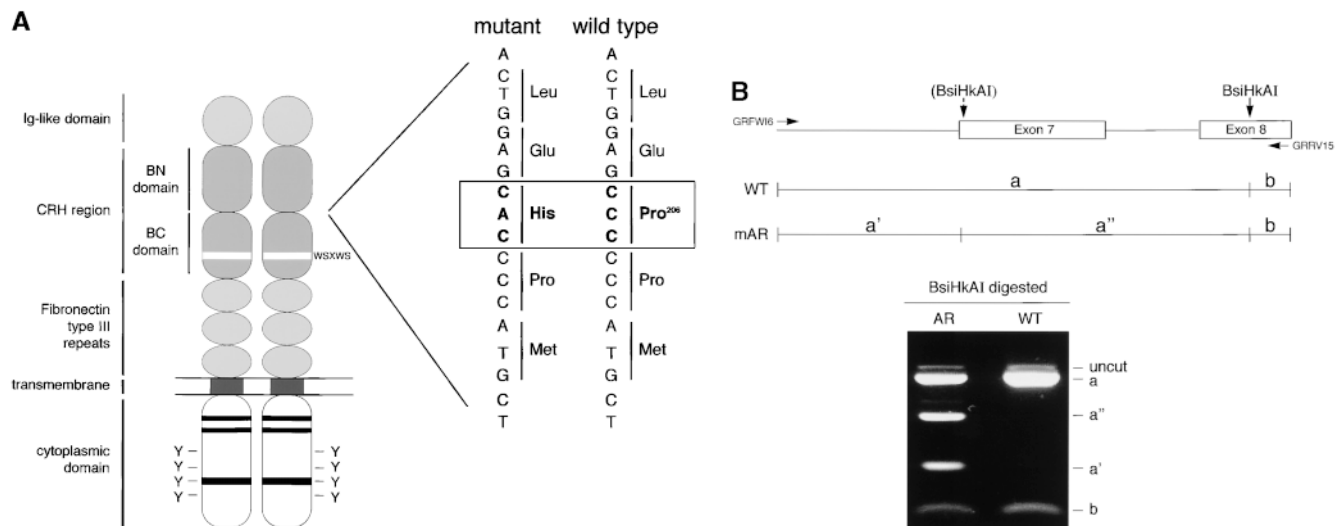


Figure 1. Identification of a point mutation in the *GCSFR* cDNA of an SCN patient hyporesponsive to G-CSF. (A) Partial nucleotide sequence of the mutant and wild-type *GCSFR* cDNA, with the relative position of the corresponding P206H mutation in the encoded G-CSF-R indicated. (B) Analysis of fibroblast chromosomal DNA for the presence of the mutation. The relevant genomic region of the *GCSFR* gene is presented (top), along with the relative positions of the primers used for PCR (GRFW16 and GRRV15). The BsiHkAI restriction sites are also indicated, with the new site introduced by the mAR mutation shown in parentheses. Digestion of a wild-type allele predictively generates two fragments, a and b, whereas digestion of a mutant allele generates three fragments, a', a'', and b, as indicated. At the bottom are the results of BsiHkAI digestion of PCR products generated from either the patient (AR) or a normal individual (WT).

duces a Pro→His substitution at position 206 (P206H) of the mature receptor (Fig. 1 A). This change lies in the joining peptide “hinge” between the NH₂- and COOH-terminal “barrels” (BN and BC domains) of the CRH region of the G-CSF-R extracellular domain (39). Along with the WSXWS sequence, this short Pro-rich stretch is highly conserved among cytokine receptors, with the position equivalent to 206 usually a Pro, or otherwise an Ala residue (12, 40). This mutation was found in ~50% of clones sequenced, indicating that essentially all bone marrow cells possessed one mutant *GCSFR* allele. This was confirmed by restriction digestion of cDNA produced in an independent RT-PCR with the enzyme BsiHkAI, as described in Materials and Methods. In addition, a fragment covering the mutation site was amplified from genomic DNA isolated from fibroblast cells of the patient. Restriction enzyme analysis with BsiHkAI revealed that both mutant and wild-type *GCSFR* alleles were also present in these cells (Fig. 1 B). Thus, unlike other *GCSFR* mutations reported (25), the mutation in patient AR is not restricted to cells of the hematopoietic system, with the patient apparently heterozygous for the mutation. Unfortunately, a full pedigree for the patient was not available to determine if the mutation was inherited, although the mother was found to possess a wild-type *GCSFR* allele and normal blood neutrophil counts (data not shown).

Myeloid 32D Cells Expressing the P206H Mutant G-CSF-R Are Hyporesponsive to G-CSF. To study the functionality of the P206H mutant G-CSF-R, designated mAR, we replaced the extracellular region of the wild-type G-CSF-R with that of the mutant form. Expression vectors containing the WT and mAR *GCSFR* cDNAs were introduced into the IL-3-dependent murine myeloid cell line 32D.cl8.6, which does not express endogenous G-CSF-R. Expression levels of the different G-CSF-R proteins in the transfectants were determined by flow cytometry using anti-G-CSF-R antiserum (Fig. 2 A). Several independent clones were obtained expressing approximately equivalent levels of wild-type and P206H mutant receptors (32D[WT] and 32D[mAR], respectively), and used in subsequent analyses. To directly test whether the P206H mutation could contribute to the G-CSF hyporesponsiveness observed in the patient, we analyzed the sensitivity of the WT and mAR clones to G-CSF by measuring DNA synthesis in ³H-TdR incorporation assays in response to titrated doses of cytokine (Fig. 2 B). This revealed a large right shift in dose-response for the 32D[mAR] clones. However, even at maximal G-CSF concentrations, 32D[mAR] clones failed to reach the same level of ³H-TdR incorporation as those expressing the wild-type receptor. These data establish that the P206H mutation causes hyporesponsiveness to G-CSF in myeloid cells.

The P206H Mutation Abrogates G-CSF-mediated Proliferation and Survival, but not Differentiation, in Myeloid 32D Cells. We next sought to determine the effect of the P206H mutation on long-term proliferation, survival, and neutrophilic differentiation responses to G-CSF. Therefore, 32D cell clones were switched from IL-3- to G-CSF-

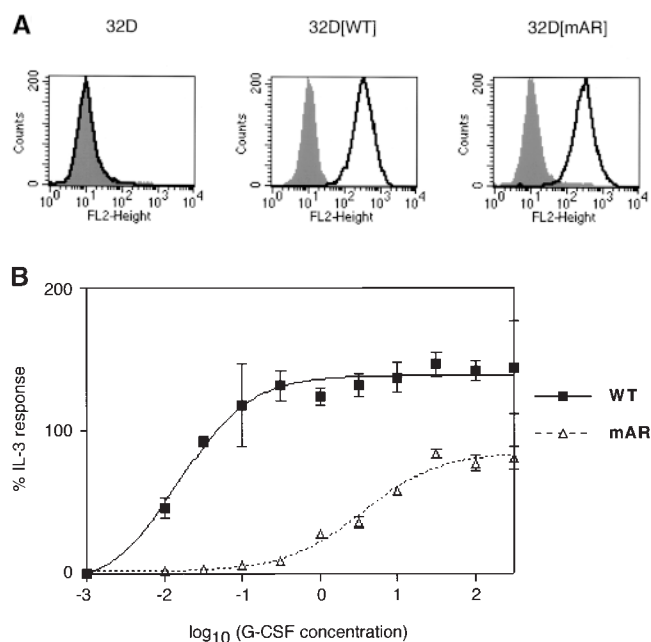


Figure 2. Expression of wild-type and P206H mutant G-CSF-Rs in myeloid 32D cells, and characterization of their G-CSF responsiveness. (A) Flow cytometric analysis of G-CSF-R expression on parental 32D.cl8.6 cells (32D) and 32D.cl8.6 cells expressing either wild-type (32D[WT]) or P206H mutant (32D[mAR]) receptors. Cells were either stained with biotinylated mouse anti-human G-CSF-R antibodies followed by PE-conjugated streptavidin, biotinylated antistreptavidin, and finally PE-conjugated streptavidin (open), or without the anti-G-CSF-R step (shaded). (B) G-CSF dose-response of representative 32D[WT] and 32D[mAR] clones in a 24-h thymidine-incorporation assay, as indicated. Data are expressed relative to the response to IL-3.

containing medium after extensive washing to remove residual IL-3. Without IL-3 or G-CSF, all transfectants died within 1–2 d and showed no signs of neutrophilic differentiation. Parental 32D.cl8.6 cells and cells transfected with empty LNCX vector also died within 1–2 d in G-CSF-containing medium. However, the 32D[WT] cells proliferated in response to G-CSF for 6–7 d (Fig. 3 A). After 6–8 d, these cells developed into terminally differentiated neutrophils, showing an enlarged cytoplasm/nucleus ratio, lobulated nuclei, and neutrophilic cytoplasm (Fig. 3 B, middle). In contrast, 32D[mAR] cells showed an almost complete block in G-CSF-mediated proliferation and reduced survival (Fig. 3 A). Since the bulk of the 32D[mAR] cells fail to survive past 6 d, it is difficult to accurately quantify the effect of the P206H mutation on differentiation. However, most 32D[mAR] cells surviving until day 7 showed clear signs of neutrophilic differentiation (Fig. 3 B, right). This suggests that differentiation signals per se are not severely affected by the receptor mutation.

Altered Signaling from the P206H Mutant G-CSF-R in Myeloid Cells. To further investigate the selective abrogation of G-CSF-mediated proliferation and survival signals by the mutant receptor, we analyzed STAT activation, since STAT3 and STAT5 have been strongly implicated in control of G-CSF-mediated differentiation and proliferation/survival, respectively (23, 24). In addition, STAT activation

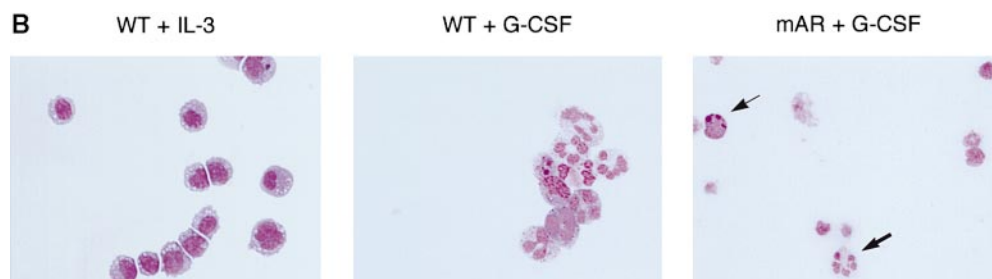
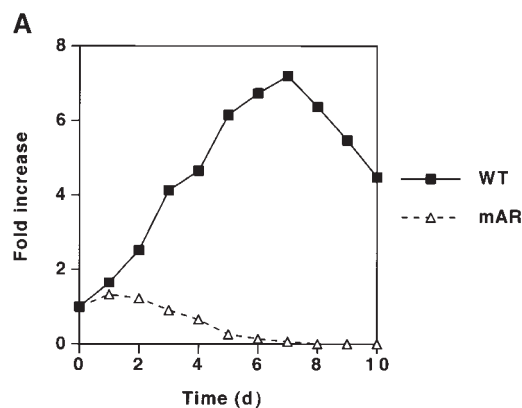


Figure 3. Effect of the P206H mutation on G-CSF-mediated growth and differentiation. (A) Cell proliferation of 32D[WT] and 32D[mAR] cells on 100 ng/ml G-CSF, as indicated. Data represent the mean growth of three clones. (B) Morphological features of representative 32D[WT] cells in the presence of IL-3, and 32D[WT] or 32D[mAR] cells after 7 d exposure to G-CSF, as indicated. For the 32D[mAR] clone, the positions of an apoptotic cell and a differentiated cell are indicated with a light and bold arrow, respectively.

represents a sensitive measure of receptor activation. This analysis revealed that, compared with 32D[WT] control cells, 32D[mAR] cells showed reduced activation of all STAT proteins and a delay in the time of peak activation from 10–15 min to 30 min, indicating defective receptor activation (Fig. 4 A). Interestingly, however, the quantitative effect on STAT1 and STAT5 activation was significantly greater than on STAT3. Examination of the dose-response of STAT activation showed a decrease in sensitivity for STAT activation from the mutant receptor, with again a larger effect on STATs 1 and 5 than on STAT3 (Fig. 4 B). We also examined activation of the Ras–mitogen-activated protein kinase (MAPK) pathway by examining extracellular signal regulatory kinase (Erk) phosphorylation. This was also both delayed and reduced in cells expressing mutant receptors (data not shown), suggesting that different pathways are altered to varying degrees by the receptor mutation.

P206H Mutant Receptors Show Reduced Ligand Binding. Given the drastic effects of the P206H mutation on both signaling pathways and biological responses from the G-CSF-R, as well as its proximity to the ligand binding sites on the receptor (41–43), we investigated whether altered ligand binding could explain our results. Scatchard analyses on 32D[WT] and 32D[mAR] clones showed that ligand binding affinity (K_d) was unaffected by the mutation (Table I). However, it was apparent in the analyses that the 32D[mAR] clones showed decreased total ligand binding. To quantitate this more precisely, we analyzed a pair of clones almost exactly matched for receptor levels as determined by FACS[®] with anti-G-CSF-R antiserum. This revealed a clear reduction in total binding sites on mAR re-

ceptors, to approximately half that on wild-type G-CSF-Rs (Table I). We also performed similar analyses on Ba/F3 clones expressing WT and mAR receptors, and obtained similar results (Table I). As a further independent test of the relative ligand binding properties of the wild-type and mutant G-CSF-Rs, we compared binding of biotinylated G-CSF to those 32D clones that expressed nearly identical levels of WT or mAR receptors by anti-G-CSF-R binding. Again there was a clear reduction in the quantitative binding of ligand to mAR receptors (Fig. 5).

Wild-type Receptors Stimulated with Nonsaturating G-CSF Concentrations Mimic the Effect of the P206H Mutation. The data above suggested that mutant mAR receptors have an altered stoichiometry of ligand–receptor complex formation, which may be responsible for their altered signaling properties. To directly test this hypothesis, we stimulated 32D[WT] cells with saturating and nonsaturating G-CSF levels, which should favor formation of different receptor complexes (see Discussion). This analysis revealed that the use of nonsaturating G-CSF concentrations (0.3 ng/ml, which is >30 times lower than the receptor K_d [44]) elicits responses that largely mimic the P206H mutant phenotype. At this concentration, cells showed decreased proliferation and survival (Fig. 6 A), although again some differentiated cells were observed (data not shown), while STAT activation, particularly of STATs 1 and 5, was decreased and delayed (Fig. 6 B).

Mutant Receptors Inhibit Signaling from Wild-type Receptors. Since the patient harbors a heterozygous mutation in the *GCSFR* gene, we sought to coexpress both wild-type and mutant receptors in the same cells in order to more accurately recapitulate the clinical situation. To achieve this, we

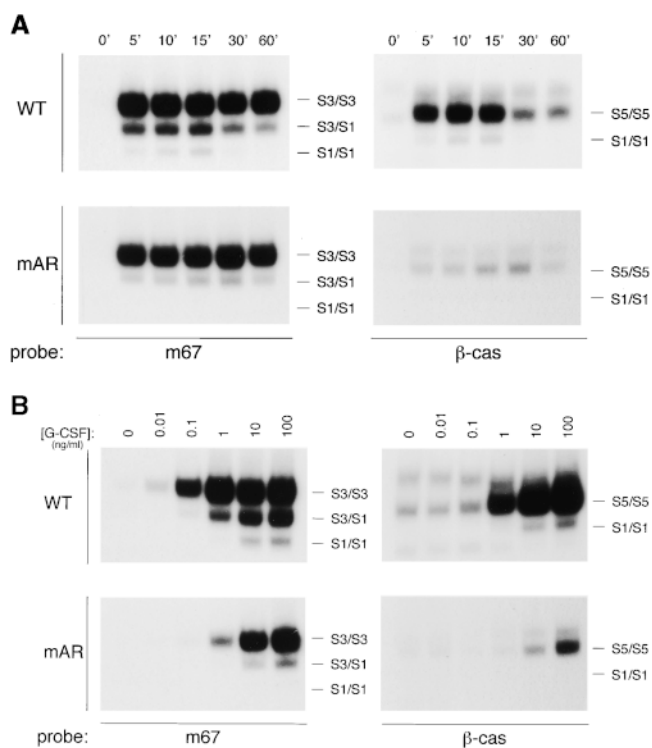


Figure 4. Effect of P206H mutation on G-CSF-mediated STAT activation. (A) Kinetics of STAT activation in 32D.c18.6 cells expressing wild-type G-CSF-R (WT) or P206H mutant (mAR) receptor. Serum- and growth factor-deprived cells were incubated at 37°C without factor or with 100 ng/ml G-CSF for the times indicated. Nuclear extracts were prepared and incubated with 32 P-labeled double-stranded m67 or β -cas oligonucleotides, as indicated. The relative position and identity of STAT complexes are shown: S1, STAT1; S3, STAT3; S5, STAT5. (B) G-CSF dose-response of STAT activation in 32D[WT] and 32D[mAR] cells as described in A, except after stimulation for 15 min with the indicated concentrations of G-CSF.

recloned both WT and mAR *GCSFR* cDNAs separately into the retroviral expression vector pBabe, which harbors a puromycin-resistance gene. Infectious retrovirus was subsequently produced using the Phoenix A amphotropic packaging line, and used to infect 32D[WT^{Neo}] cells, which express the wild-type G-CSF-R from the neomycin-resistance encoding pLNCX vector. Subsequent bulk selection on puromycin yielded cells expressing just wild-type receptor (32D[WT^{Neo}/WT^{Puro}] cells) or coexpressing wild-type and mutant receptors (32D[WT^{Neo}/mAR^{Puro}] cells). FACS[®] analysis of these bulk cultures revealed an increase in G-CSF-R expression due to the introduction of the pBabe retrovirus (Fig. 7 A). Northern blot analysis confirmed the presence of *GCSFR* transcripts from both pLNCX and pBabe expression vectors (data not shown). When these cultures were switched from IL-3 to G-CSF, the 32D[WT^{Neo}/WT^{Puro}] cells showed substantially more proliferation than 32D[WT^{Neo}/mAR^{Puro}] cells, especially at low G-CSF concentrations (Fig. 7 B). Finally, analysis of G-CSF-induced STAT5 activation in these cells showed that the presence of the mutant receptor also suppressed the

Table I. Scatchard Analysis of Clones Expressing Wild-type (WT) and P206H Mutant (mAR) G-CSF-Rs

Cell line	Receptor type	K_d (mean, $n = 3$)	Sites/cell (matched)
		<i>pM</i>	
32D	WT	6.5×10^2	460
	mAR	4.4×10^2	230
Ba/F3	WT	8.0×10^2	510
	mAR	5.2×10^2	200

wild-type receptor signal in this regard, producing an altered dose-response of activation (Fig. 7 C).

Discussion

SCN is a heterogeneous disorder defined by a severe reduction in blood neutrophil count—the result of a maturation arrest at the promyelocyte/myeloid stage defect in the bone marrow (1–3). The molecular mechanisms leading to SCN remain largely unknown. However, G-CSF treatment can restore granulopoiesis in the majority of SCN patients, which suggests that defects in G-CSF signal transduction might be important in the etiology of the disease. Indeed, in a subset of these G-CSF-responsive SCN patients, mutations have been identified in the cytoplasmic domain of the G-CSF-R that generate truncated receptors defective in G-CSF maturation signaling (25–28, 34). Although these mutations correlate well with leukemic progression from SCN, their exact contribution to neutropenia is still controversial. The mutation described in this report represents a novel G-CSF-R mutation affecting the extracellular domain of the receptor in an SCN patient who was hyporesponsive to G-CSF treatment. Introduction of this P206H mutant receptor into myeloid cells reproduces the hyporesponsiveness to G-CSF, severely affecting proliferation and survival, although not differentiation, responses to G-CSF. These data further highlight the

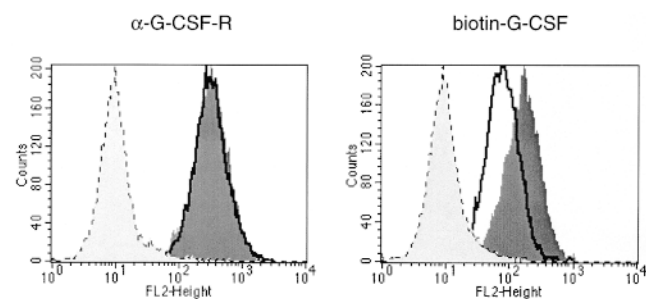


Figure 5. Binding of G-CSF to wild-type and P206H mutant receptors. FACS[®] analysis of 32D parental (light gray shaded, dotted line), 32D[WT] (dark gray shaded, no line), or 32D[mAR] (open, bold line) clones with either α -G-CSF-R antiserum or biotinylated G-CSF, as indicated.

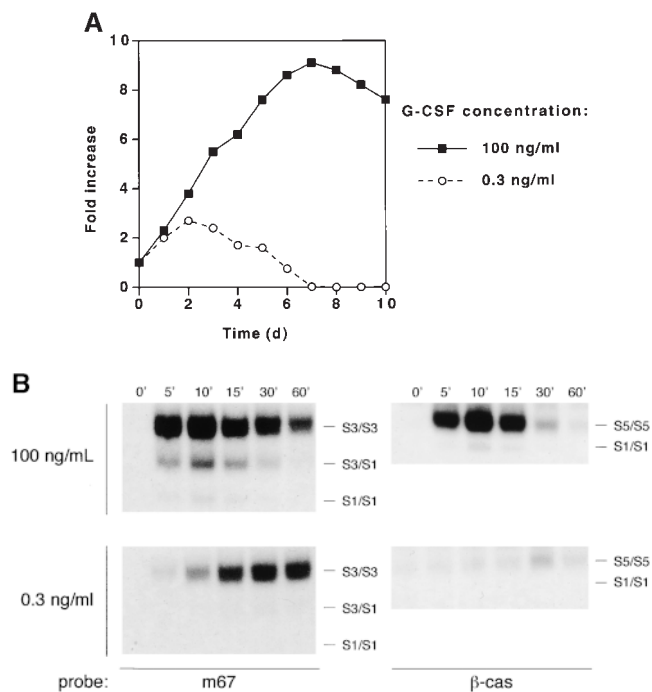


Figure 6. Analysis of signaling from the wild-type G-CSF-R at saturating and nonsaturating G-CSF concentrations. (A) Growth of 32D[WT] cells at either 100 or 0.3 ng/ml G-CSF, as indicated. (B) STAT activation in 32D[WT] cells at either 100 or 0.3 ng/ml G-CSF, as indicated, performed as described in the legend to Fig. 4 A.

importance of *GCSFR* mutations in the pathophysiology of SCN, and also for the first time implicate such mutations in hyporesponsiveness to G-CSF therapy. In addition, the mutation also severely, but selectively, abrogates signaling from the G-CSF-R, apparently due to altered ligand-receptor complex formation, which contributes to our understanding of cytokine receptor function.

How does the P206H mutation lead to the observed defects in receptor function? It is clear that the mutation affects ligand-receptor stoichiometry, such that the number of ligand binding sites per receptor is reduced, suggesting that the mutation alters the molecular architecture of the receptor complex. Using purified G-CSF-R, Fukunaga et al. have shown that high-affinity G-CSF-Rs consist of oligomers (44). From studies with isolated recombinant domains, it appears that G-CSF can bind to the isolated BN and BC subdomains of the G-CSF-R CRH region in a 1:1 stoichiometry (42, 43). However, unlike other receptors, the G-CSF-R requires its Ig domain in addition to its CRH domain for high-affinity binding and oligomerization (45, 46). From these and other studies, Hiraoka et al. have proposed a model for the interaction of G-CSF with its receptor—at low ligand concentrations, an asymmetric 2:1 receptor-ligand complex is formed, whereas at high G-CSF concentrations, this converts to a 4:4 receptor-ligand tetramer (45). In contrast, Horan et al. have implicated a conversion instead from a 2:1 to a 2:2 receptor-

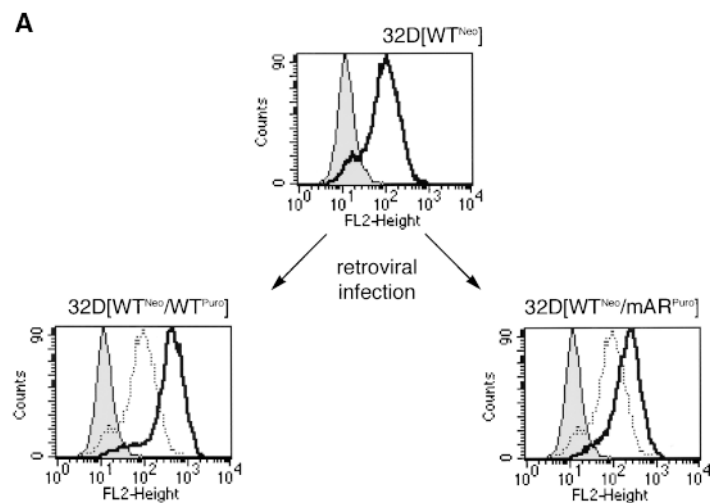
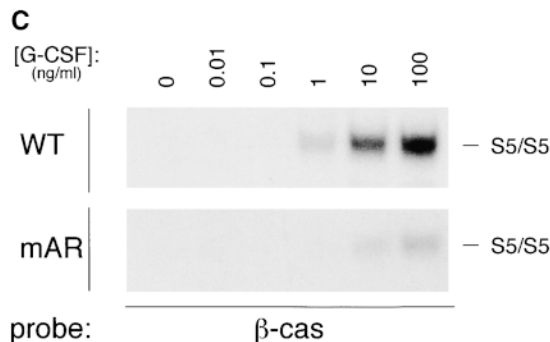
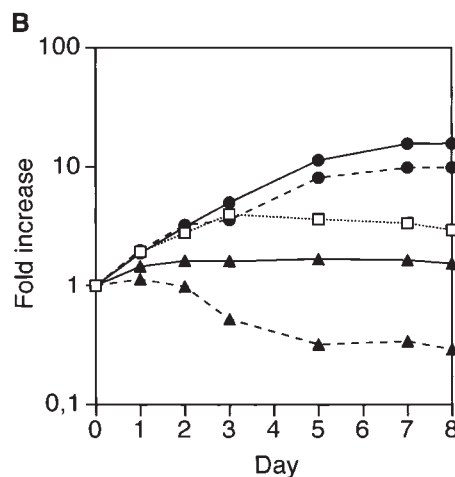


Figure 7. Analysis of 32D cells coexpressing wild-type and mutant receptors. (A) FACS[®] analysis of 32D[WT^{Neo}], 32D[WT^{Neo}/WT^{Puro}], and 32D[WT^{Neo}/mAR^{Puro}] cells with α -G-CSF-R antiserum, as described in the legend to Fig. 2 A. In the bottom panels, the dotted line represents the G-CSF-R expression level of the original 32D[WT^{Neo}] clone. (B) Growth of 32D[WT^{Neo}/WT^{Puro}] cells (filled circles) or 32D[WT^{Neo}/mAR^{Puro}] cells (filled triangles) at G-CSF concentrations of either 100 ng/ml (solid line) or 1 ng/ml (dashed line). The growth curve of the original 32D[WT^{Neo}] clone at 100 ng/ml G-CSF is included for comparison (open squares, dotted line). (C) G-CSF-induced STAT5 activation in 32D[WT^{Neo}/WT^{Puro}] cells (WT) or 32D[WT^{Neo}/mAR^{Puro}] cells (mAR), as described in the legend to Fig. 4 B.



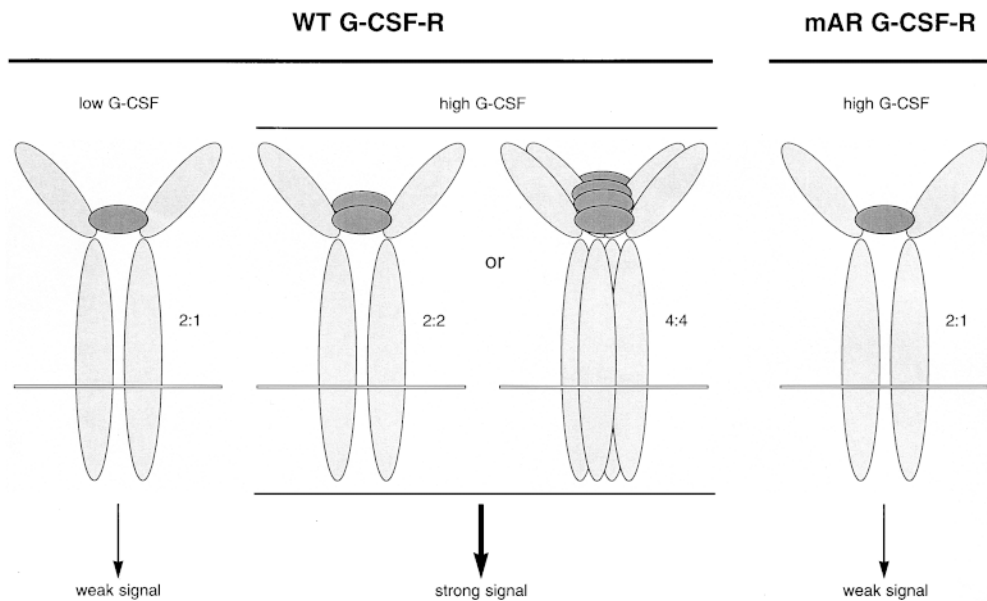


Figure 8. Model for receptor-ligand complexes formed with WT and mAR (P206H) mutant G-CSF-Rs.

ligand complex depending on G-CSF concentration (47, 48). Regardless of the exact composition, this ligand concentration-dependent transition to a higher order complex involving the Ig domain contrasts with other hematopoietin receptors, such as growth hormone receptor, which only requires its CRH region to form a simple, symmetrical 2:1 receptor-ligand complex (13, 49). In addition, we have recently shown that the ligand sensitivity for STAT3 activation is ~ 1 log higher than for activation of STATs 1 and 5 from the wild-type G-CSF-R (22). This suggests that different intracellular signaling complexes are also formed depending on ligand concentration: at low concentrations, one that can activate STAT3, and at high concentrations, one that can activate all three STATs.

The P206H mutation we have identified lies in a short connecting loop or hinge between the BN and BC subdomains of the CRH region (39). From the structures of the CRH region from the closely related gp130 receptor component (50) and the isolated BC domain of the G-CSF-R (51), we can expect this mutation to impact on the β -sheet angles as well as on the relative orientation of the BN and BC domains. In addition, we have shown that this mutation leads to a reduction in ligand binding sites, and produces a drastic effect on signaling and biological responses. In light of this data and the studies detailed above, we would propose the following model for the signaling complexes formed with wild-type and P206H mutant receptors at different ligand concentrations (Fig. 8). At low ligand concentration, wild-type receptors form a 2:1 complex that can activate a subset of signaling pathways, including STAT3 (weak signal). At high ligand concentration, there is a shift to a higher order complex (2:2 or 4:4), which can activate all pathways, including STATs 1 and 5 (strong signal). In contrast, the mAR receptor is able to form the asymmetric 2:1 complex normally, but the P206H mutation perturbs the receptor in a manner that blocks higher order binding. This would explain the 50% reduction in

binding sites per mutant receptor in spite of an unaltered receptor K_d , as well as the ability of the mutant to elicit only weak intracellular signals (such as STAT3), i.e., equivalent to the complex formed when the wild-type receptor is stimulated at low G-CSF concentration, with similar biological outcomes. Consistent with this model, a G-CSF-R mutant in which the Ig domain was deleted, which could presumably form a 2:1 but not a higher order complex, also retained a very weak signal transducing ability (41).

Recently, the X-ray structure of the EpoR extracellular domain has been solved (52, 53). These studies have revealed that the EpoR is present as a dimer in the absence of ligand, with ligand binding inducing a conformational change of the dimer necessary for signal transduction. Importantly, this is accompanied by an alteration in the interdomain angle between the BN and BC subdomains. Our data are consistent with this model, and further suggest that there may indeed be a two-step conformational change in the G-CSF-R as more ligand binds to the receptor complex, the latter of which is impaired by the P206H mutation, which lies in a prime position to perturb the interdomain angle.

Our suggestion of a ligand concentration-dependent switch in complex formation of the wild-type receptor may also have important consequences for understanding the control of basal versus “stress” granulopoiesis from the G-CSF-R. Recent data have implicated STAT3 activation in the control of differentiation from the G-CSF-R (22, 23), whereas STAT5 seems important in the control of G-CSF-mediated proliferation and survival (24). Thus, we would envisage a switch from a differentiation function (STAT3) at basal G-CSF levels, to a proliferative/survival function (STAT5, and other pathways?) when G-CSF levels are elevated to facilitate a rapid expansion of the granulocytic compartment, such as in response to infection.

The identification of a mutation in the extracellular domain of the G-CSF-R in an SCN patient unresponsive to

normal G-CSF treatment has important ramifications. First, PCR screening for *GCSFR* mutations in SCN is currently confined to the cytoplasmic region of the receptor. Our data suggest the need for a more complete analysis, particularly in "nonresponders" to G-CSF. Second, as our understanding of the biological consequences of different

G-CSF-R or other mutations in SCN increases, it would seem appropriate in the future to prescribe different treatments to different categories of SCN patients. In this regard, it will be of interest to determine whether the use of prednisone in combination with G-CSF may be of benefit for other patients hyporesponsive to standard G-CSF therapy.

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