Point Mutations in the Conserved Box 1 Region Inactivate the Human Granulocyte Colony-Stimulating Factor Receptor for Growth Signal Transduction and Tyrosine Phosphorylation of p75c-rel

By Belinda R. Avalos, Melissa G. Hunter, Jennifer M. Parker, Sarah K. Ceselski, Brian J. Druker, Seth J. Corey, and Veela B. Mehta

The human granulocyte colony-stimulating factor receptor (hG-CSFR) belongs to the cytokine receptor superfamily. As with other members of this family, the cytoplasmic domain of hG-CSFR lacks intrinsic tyrosine kinase activity. To identify critical regions mediating growth signal transduction by hG-CSFR, deletions or site-directed amino acid substitutions were introduced into the cytoplasmic domain of hG-CSFR, and the mutant cDNAs were transfected into the murine interleukin-3 (IL-3)-dependent Ba/F3 and FDCP cell lines. Truncation of the carboxy-terminal end of the receptor to the membrane-proximal 53 amino acids of the cytoplasmic domain, which retained the conserved Box 1 and Box 2 sequence motifs, decreased the ability of hG-CSFR to transduce G-CSF-mediated growth signals without an associated loss in receptor binding affinity. Substitution of proline by alanine at amino acid positions 639 and 641 within Box 1 completely abolished the G-CSF-mediated growth signal. Rapid induction of tyrosine phosphorylation of several cellular proteins, including a 75-kD protein (p75) identified as c-rel, was an early event associated with transduction of proliferative signals by hG-CSFR in Ba/F3 transfectants. Mutant receptors containing Pro-to-Ala substitutions that inactivated the receptor for mitogenic activity also inactivated the receptor for tyrosine-specific phosphorylation of p75.

These results show that the conserved Box 1 sequence motif (amino acids 634 to 641) is critical for mitogenesis and activation of cellular tyrosine kinases by hG-CSFR.

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MATERIALS AND METHODS

Cells and cell culture. The murine pro-B cell line Ba/F3 was grown in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 10% WEHI conditioned media as a source of IL-3. Ba/F3 and WEHI cells were provided by Dr H. Lodish (Massachusetts Institute of Technology, Cambridge, MA). The murine myeloid precursor cell line FDCP mix was provided by Dr Neil Wilkie (Ohio State University, Columbus, OH) and was grown in Fischer’s media (GIBCO) supplemented with 10% horse serum and 10% WEHI conditioned media.

Antisera. Anti-phosphotyrosine monoclonal antibody 4G10 was obtained from Upstate Biotechnology Inc (UBI, Lake Placid, NY). Rabbit anti-c-rel antiserum generated against an epitope corresponding to 300 amino acids mapping within the amino terminus of human c-rel p75 was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Construction of expression plasmids and site-directed mutagenesis. The alternatively spliced hG-CSFR D7 cDNA insert, a gift from Dr A. Larsen (Immunex Corp, Seattle, WA), was excised from the bluescript sk+ plasmid by EcoRI and XbaI digestion. Nonpalenomic BstXI linkers (Invitrogen, San Diego, CA) were ligated to

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the blunt-ended 2.6-kb cDNA fragment and subcloned into the BsuXI site of the eukaryotic expression vector pCDM8 (a gift from Dr B. Seed, Massachusetts Institute of Technology) to obtain the wild-type hG-CSFR (pCD-WT).

Deletion mutants of hG-CSFR were generated as follows: pCD-WT was digested with Mse I or Cfr101, corresponding to nucleotide (nt) 2442 and nt 2282, respectively, blunt-ended with the Klenow fragment of Escherichia coli, gel-purified, and ligated to a universal translation terminator and then to BsuXI linkers. Both were then digested with Xho I, blunt-ended, gel-purified, and subcloned into BsuXI and blunt-ended HindIII sites of pCDM8 to generate pCD-Mse and pCD-Cfr, respectively.

Construction of the cytoplasmic deletion mutant was performed by recombinant polymerase chain reaction (PCR) using the following four oligonucleotide primers: PF1, 5' AGGAAGAATGCCCTCTGGCTACGTCACCTTCCA 3'; PF2, 5' GGCGCCGAATTCTGCGGGGCAATTCCTGCTCTGCGGATCTTCCA 3'; PR1, 5' GGCCGCAATTCTGCGGGGCAATTCCTGCTCTGCGGATCTTCCA 3'; PR2, 5' ATACACGCTCAGCTGCACCTTCA 3'. Primer PF1 is a sense primer to the cytoplasmic domain of hG-CSFR (nt 2124 to nt 2139), and PR2 is an antisense primer containing 17 nucleotides of the PR sequence (underscored) and 16 nucleotides of the pCDM8 vector (nt 2599 to nt 2615). PR3 is an antisense primer containing 22 nucleotides of the pCDM8 vector (nt 2776 to nt 2797). In step 1 PCR amplification, the extracellular portion of hG-CSFR was amplified using PF1 and PR1, and the region from pCDM8 was amplified using PF2 and PR2 oligonucleotide primers. As a template, a pCD-WT PCR product was digested with Xho I and Xho I, gel-purified, and ligated into the same sites of pCD-WT to generate pCD-cyto. The universal translation terminator sequence was introduced into the blunt BsuXI restriction site.

Site-specific mutations of hG-CSFR were performed by primer-mediated, site-directed mutagenesis using PCR. Oligonucleotide primers were as follows: PF1, 5' ACACCGCTCAGCTGCACCTTCCA 3'; PF2, 5' GATACGGAATTCTGCGGGGCAATTCCTGCTCTGCGGATCTTCCA 3'; PR1, 5' ATACACGCTCAGCTGCACCTTCA 3'; and PR2, 5' ATACACGCTCAGCTGCACCTTCA 3'. Primer PF1 is a sense primer in the external region of hG-CSFR (nt 1337 to nt 1357). PR1 is an antisense primer containing Not I, EcoRI, and BsuWI restriction enzyme recognition sequences (underscored) and 16 nucleotides (nt 2018 to nt 2123) from the transmembrane domain of hG-CSFR. PF2 is a sense primer containing 17 nucleotides of the PR sequence (underscored) and 16 nucleotides of the pCDM8 vector (nt 2599 to nt 2615). PR3 is an antisense primer containing 22 nucleotides of the pCDM8 vector (nt 2776 to nt 2797). In step 1 PCR amplification, the extracellular portion of hG-CSFR was amplified using PF1 and PR1, and the region from pCDM8 was amplified using PF2 and PR2 oligonucleotide primers. As a template, a pCD-WT PCR product was digested with EcoRI and blunt-ended, gel-purified, and subcloned into the BsuWI restriction site of pCDM8.

Primer PF1 is a sense primer containing 17 nucleotides of the PR sequence (underscored) and 16 nucleotides of the pCDM8 vector (nt 2599 to nt 2615). PR3 is an antisense primer containing 22 nucleotides of the pCDM8 vector (nt 2776 to nt 2797). Deletion mutants of hG-CSFR were generated as follows: pCD-WT was digested with Mse I or Cfr101, corresponding to nucleotide (nt) 2442 and nt 2282, respectively, blunt-ended with the Klenow fragment of Escherichia coli, gel-purified, and ligated to a universal translation terminator and then to BsuXI linkers. Both were then digested with Xho I, blunt-ended, gel-purified, and subcloned into BsuXI and blunt-ended HindIII sites of pCDM8 to generate pCD-Mse and pCD-Cfr, respectively.

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BOX 1 POINT MUTATIONS INACTIVATING THE G-CSFR

A

- **Ex**
- **TM**
- **Cy**

**pCD-WT**

**pCD-Mse**

**pCD-Cfr**

**pCD-Cyto**

B

**4PR**

**2PR**

- **R**
- **K**
- **N**
- **P**
- **L**
- **W**
- **S**
- **V**
- **P**
- **D**
- **P**
- **A**
- **H**
- **S**

**Clones**

**Immunocomplexes**

**Scatchard analysis**

**Table 1. Binding Properties of Ba/F3 Transformants Expressing hG-CSFR cDNAs**

<table>
<thead>
<tr>
<th>Clone</th>
<th>(K_d) (pmol/L)</th>
<th>Sites/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>B/Wt</td>
<td>40-838</td>
<td>279-3,120</td>
</tr>
<tr>
<td>B/RWT</td>
<td>19-185</td>
<td>178-6,310</td>
</tr>
<tr>
<td>B/Mse</td>
<td>157-529</td>
<td>2,195-7,702</td>
</tr>
<tr>
<td>B/Cfr</td>
<td>105-320</td>
<td>1,526-8,100</td>
</tr>
<tr>
<td>B/Cyto</td>
<td>55-761</td>
<td>3,039-14,816</td>
</tr>
<tr>
<td>B/4PR</td>
<td>18-402</td>
<td>77-662</td>
</tr>
<tr>
<td>B/2PR</td>
<td>52-580</td>
<td>234-2,929</td>
</tr>
</tbody>
</table>

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**RESULTS**

**Requirement of the membrane-proximal 53 amino acids of the cytoplasmic domain of hG-CSFR for G-CSF-induced mitogenesis.** Expression of either of two forms of hG-CSFR that share the first 96 amino acids of the cytoplasmic domain and differ only at the carboxyl terminus has previously been shown to render BA.F/B03 cells responsive to G-CSF.\(^{28}\) To extend these observations, a series of deletion mutants carrying carboxy-terminal truncations at amino acid positions 735, 682, and 630 were constructed and introduced into the IL-3-dependent Ba/F3 and FDCP cell lines. The mutant hG-CSFR cDNAs were generated by introduction of universal translation terminator codons at the indicated positions and cloned into the eukaryotic expression vector pCDM8. The resultant truncated hG-CSFR cDNAs (Fig 1A) were designated pCD-Mse (aa735), pCD-Cfr (aa682), and pCD-cyto (aa630); pCD-Mse retains the membrane-proximal Box 1 and Box 2 sequences and an additional 54 amino acids of the D7 (class IV) receptor isoform; pCD-Cfr terminates just outside the conserved Box 2 sequence; and pCD-cyto is devoid of the entire cytoplasmic domain. The wild-type hG-CSFR variant D7 cDNA\(^7\) cloned into pCDM8 and designated pCD-WT as well as truncated hG-CSFR cDNAs were cotransfected with the p309 plasmid carrying the neomycin resistance gene into Ba/F3 and FDCP cells.

We initially examined the G-CSF binding properties of these stable transformants by \(^{125}\)I–G-CSF binding assays. The parental Ba/F3 and FDCP cells did not bind G-CSF, nor did these cells when transfected with the pCDM8 and p309 neo vectors alone (data not shown). Scatchard analysis of the equilibrium binding data (data not shown) indicated that Ba/F3 clones transformed with the wild-type D7 hG-CSFR (B/Wt) cDNA and truncated hG-CSFR cDNAs (B/Mse, B/Cfr, B/cyto) expressed a single class of high affinity receptors with dissociation constant (\(K_d\)) values of 19 to 838 pmol/L (Table 1). Similar results were obtained with FDCP-derived stable transformants (data not shown). The binding properties for mutant transformants expressing truncated receptors were similar to those expressing the B/Wt cDNA and also were consistent with previous results reported with BA.F/B03, FDCP-1, and B9 cells transfected with murine and human G-CSFR cDNAs.\(^{13,30}\) Interestingly, Scatchard analysis (data not shown) of the binding of \(^{125}\)I–G-CSF to three independent B/cyto clones demonstrated a higher level of receptor expression than observed in wild-type transformants, the reasons for which are not known. Northern blot analysis of total RNA isolated from wild-type D7 and truncated hG-CSFR transformant clones using the hG-CSFR mRNA species (Fig 2A). An approximately 2.6-kb band was detected. As expected, the cyto mutants expressed smaller mRNA transcripts. Crosslinking experiments confirmed that the transformant clones expressed hG-CSFR binding proteins. A ligand-receptor complex with an apparent molecular weight of 150 to 160 kD was observed, and as expected, a smaller crosslinked complex was seen with the B/cyto mutant (Fig 2B). No mRNA transcripts or crosslinked complexes for hG-CSFR were detected in the control trans-
formant clones B/CDN and F/CDN transfected with pCDM8 and p309 neo vectors alone (Fig 2A and B).

The ability of the truncated receptor mutants to transduce G-CSF–mediated growth signals was examined by [3H]thymidine uptake and long-term cell growth. As shown in Figs 3 and 4, control transformants B/CDN and F/CDN did not respond to G-CSF as expected. B/WT and F/WT clones expressing wild-type D7 hG-CSFR responded to G-CSF in a dose-dependent manner, although concentrations of G-CSF exceeding 2 nmol/L were slightly inhibitory in the B/WT transformant. The maximum [3H]thymidine uptakes for B/WT and F/WT were obtained at 200 pmol/L and 20 nmol/L G-CSF, respectively (Fig 3). A concentration of 100 pmol/L G-CSF was sufficient to support long-term growth of wild-type transformants (Fig 4). Mse mutants terminating at residue 735 also responded to G-CSF in a concentration-dependent manner up to 2 nmol/L for B/Mse and 20 nmol/L for F/Mse. Long-term growth of both transformants was observed with 100 pmol/L G-CSF. Although the Cfr mutant expressed in Ba/F3 or FDCP cells (B/Cfr, F/Cfr) responded to G-CSF in a dose-dependent manner, growth of this mutant was 40% to 60% less than that observed with transformants transfected with the wild-type D7 hG-CSFR cDNA (B/Wt, F/Wt). Cfr mutants also required higher concentrations of G-CSF to stimulate DNA synthesis at levels comparable with wild-type D7 transformants. Deletion mutants lacking the entire hG-CSFR cytoplasmic domain (B/cyto, F/cyto) showed no response to G-CSF, either by [3H]thymidine uptake or long-term growth (Figs 3 and 4).
BOX 1 POINT MUTATIONS INACTIVATING THE G-CSFR

These results indicate that although the cytoplasmic domain of hG-CSFR is not required for G-CSF binding and formation of high affinity receptors for G-CSF, it is essential for signal transduction. The membrane-proximal 53 amino acids of the cytoplasmic domain are critical for growth signal transduction by hG-CSFR, and additional amino acids are required for induction of maximal proliferative responses.

Point mutations in the proline-rich region of Box 1 inactivate hG-CSFR for growth signal transduction. The conserved Box 1 and Box 2 motifs\textsuperscript{22} lie within the membrane-proximal 53 amino acids of the cytoplasmic domain of hG-CSFR. As the Box 1 region of hG-CSFR is rich in proline...
residues and possesses a Pro-Xaa-Pro (PXP) motif that is present in most members of the cytokine receptor family, we introduced site-specific mutations by primer-mediated mutagenesis using PCR to determine the functional significance of the proline residues within Box 1 in hG-CSFR–mediated growth signal transduction.

Initially, all three proline residues within Box 1 as well as the one located just outside Box 1 (Pro633, Pro636, Pro639, and Pro641) were substituted with alanine (Fig 1B). The resultant mutant plasmid pCD-4PR containing the entire D7 hG-CSFR cytoplasmic domain except for these four alanine substitutions was transfected into Ba/F3 and FDCP cells, and the stable transformants B/4PR and F/4PR were established. Expression of hG-CSFR by these stable transformants was confirmed by [125I]–G-CSF binding assays (Table 1), Northern blot analysis (Fig 2A), and crosslinking (Fig 2B). Although 4PR receptor mutants bound G-CSF with similar affinities as the wild-type D7 receptor (Table 1), these mutants did not transduce G-CSF–mediated DNA synthesis (Fig 3) or long-term growth (Fig 4). Even high concentrations of G-CSF up to 200 nmol/L could not support the growth of these mutants (Fig 4). These results were confirmed using three independent B/4PR clones.

We further examined which of the four proline residues were essential for G-CSF–induced mitogenesis. In the conserved PXP sequence, Pro639 and Pro641 were substituted with alanine (Fig 1C) and the mutant transformants B/2PR and F/2PR were established in Ba/F3 and FDCP cells. Alanine substitution for these two proline residues completely inactivated the receptor for either [3H]thymidine incorporation or long-term growth in G-CSF (Figs 3 and 4). These results were confirmed in three independent B/2PR clones. Expression of the receptor by these mutants was confirmed by Northern blot analysis (Fig 2A) and affinity crosslinking (Fig 2B). Scatchard analysis of the equilibrium binding of [125I]–G-CSF to these mutant transformants (data not shown) was consistent with expression of a single class of high affinity receptors, with a kd similar to that observed for transformants expressing wild-type D7 hG-CSFR (Table 1). Although both 4PR and 2PR transformants tended to express fewer receptors than wild-type D7 transformants (Table 1), the failure of these mutants to transduce mitogenic signals in response to G-CSF could not be attributed to their slightly lower level of receptor expression, as there was no direct correlation between the level of receptor expression and magnitude of proliferative response (data not shown). Thus, the PXP sequence in the conserved Box 1 region of the cytoplasmic domain of hG-CSFR is essential for hG-CSFR–mediated mitogenesis.

**Induction of tyrosine-specific phosphorylation of cellular substrates by hG-CSFR.** Tyrosine phosphorylation of cellular substrates has been reported for most of the cytokine receptors studied. Recently, tyrosine phosphorylation of c- rel has been reported in human neutrophils after stimulation with G-CSF. Increased tyrosine phosphorylation of c- rel was shown to be unique for G-CSF and was not observed after treatment of neutrophils with other cytokines including GM-CSF and tumor necrosis factor (TNF). Therefore, we examined the ability of Ba/F3 cells transfected with the wild-type D7 hG-CSFR cDNA (B/WT) to induce tyrosine phosphorylation of cellular substrates after stimulation with G-CSF.

![Fig 5. Tyrosine-specific phosphorylation of cellular proteins in Ba/F3 cells expressing D7 hG-CSFR splice variant stimulated with G-CSF. Transformants were deprived of serum and WEHI-CM for 8 hours and then incubated in the absence (−) or presence (+) of 10 ng/mL G-CSF for 10 minutes at 37°C. Cell lysates were prepared and analyzed by SDS-PAGE. Immunoblot analysis was performed with the 4G10 anti-phosphotyrosine antibody. The size of tyrosine-phosphorylated proteins is indicated on the right.](http://www.bloodjournal.org)

Immunoblot analysis of cell lysates from B/WT transformants stimulated with 10 ng/mL G-CSF using the 4G10 anti-phosphotyrosine antibody demonstrated tyrosine phosphorylation of cellular proteins of 150, 130, 105, 91, 75, 59, 47, 44, and 37 kD, among which the 150-, 105-, 75-, and 37-kD phosphoproteins were most prominent (Fig 5). Although increased tyrosine phosphorylation over basal levels was observed for some of the cellular proteins, the 75-kD protein (p75) was most prominently inducibly phosphorylated. Tyrosine phosphorylation after G-CSF stimulation was rapid and maximal at 10 minutes and decreased thereafter. By 60 minutes, the majority of the phosphorylated substrates could not be detected (data not shown). These results indicate that rapid phosphorylation of tyrosine residues on a multitude of intracellular proteins is an early event after ligand binding to hG-CSFR.

**Tyrosine phosphorylation of p75 is coupled with hG-CSFR–mediated mitogenesisis.** We then examined the ability of Ba/F3 cells expressing hG-CSFR mutant cDNAs to induce tyrosine phosphorylation of cellular substrates. The profile of tyrosine phosphorylated proteins for each mutant transformant is shown in Fig 6A. As shown in Fig 6B, phosphorylation of p75 was observed only in cells expressing...
mutant receptors that were capable of transducing mitogenic signals in response to G-CSF (Mse and Cfr). In contrast, cells expressing mutant receptors that did not transduce G-CSF proliferative signals lacked the ability to induce phosphorylation of p75 (cyto, 4PR, and 2PR). Substitution of the two proline residues alone in the conserved PXP sequence of Box 1 with alanine was sufficient to inactivate hG-CSFR for induction of tyrosine phosphorylation of p75. Interestingly, the level of tyrosine phosphorylation of p75 appeared reduced in the Cfr mutant, which also showed a decreased capacity for growth in response to G-CSF. These results indicate that tyrosine phosphorylation of p75 is an early event associated with transduction of mitogenic signals by hG-CSFR and that proline residues within the PXP sequence motif of Box 1 are required for induction of tyrosine phosphorylation of p75.

Identification of p75 as c-rel. Based on the recent observation that c-rel is rapidly and specifically tyrosine phosphorylated in human neutrophils after G-CSF stimulation, we examined whether the 75-kD protein that is inducibly tyrosine phosphorylated in Ba/F3 cells expressing the wild-type D7 hG-CSFR cDNA (B/WT) is also c-rel. Immunoprecipitation of G-CSF–stimulated B/WT cell lysates with c-rel antibody followed by immunoblotting with 4G10 demonstrated that c-rel is inducibly tyrosine phosphorylated by G-CSF (Fig 7A). Immunoblotting using c-rel–specific antibody of cell lysates from G-CSF–stimulated B/WT cells immunoprecipitated with 4G10 confirmed that the tyrosine-phosphorylated 75-kD protein is c-rel (Fig 7B). To demonstrate that c-rel is the major component of the 75-kD band, lysates were precleared with anti-c-rel antiserum by immunoprecipitation, and the unbound fraction was subjected to immunoprecipitation with 4G10. Immunoblot analysis with 4G10 of G-CSF–stimulated B/WT cell lysates immunoprecipitated with c-rel antibody yielded an inducibly tyrosine-phosphorylated band at 75 kD (Fig 8A) that was not apparent when the unbound (precleared) fraction was immunoprecipitated with 4G10 and subjected to 4G10 immunoblotting (Fig 8B).

**DISCUSSION**

Recently, distinct regions in the cytoplasmic domain of the G-CSFR that mediate proliferation and differentiation signals have been identified. The signal transduction pathways involved in generation of a proliferative response by hG-CSFR and the critical amino acids of hG-CSFR responsible for interaction with growth signal transducing molecules have not been elucidated. Mutational analyses of the mouse G-CSFR cytoplasmic domain have indicated that the Box 1 motif, which is conserved in other members of the cytokine receptor superfamily, is important for G-CSF–mediated growth signaling. Replacement of Pro 638 and Pro 640 within the Box 1 PXP motif, as well as Trp634 and Asp 639, with alanine has been reported to inactivate the mouse receptor for G-CSF–triggered growth signal transduction.
In the present study, we show that the PXP sequence within the conserved Box 1 motif of hG-CSFR is indispensable for growth signaling. Substitution alone of Pro639 and Pro641 by alanine completely abolished growth signal transduction by hG-CSFR. The loss of signal-transducing ability by hG-CSFR coincided with disappearance of tyrosine-specific phosphorylation of the transcriptional regulator p75<sup>corel</sup> in Ba/F3 transformants. Our data suggest that the PXP motif of Box 1 of hG-CSFR is critical for activation of a tyrosine kinase, which appears to directly transduce a signal to a protein that functions as a transcriptional regulator.

In a previous report, substitution of the same proline residues in the PXP motif of Box 1 of gp130 with serine was shown to result in the loss of signal-transducing capacity by gp130 and an associated disappearance of tyrosine phosphorylation of gp130 and Jak2. The mechanism by which substitution of these two critical proline residues alone in hG-CSFR and gp130 results in inactivation of these receptors for mitogenic activity and protein tyrosine phosphorylation is unknown at present. It is possible that substitution of the two proline residues changes the tertiary structures of the cytoplasmic regions of hG-CSFR and gp130, as proline is a strong helix breaker and believed to be important in protein structure. An alternative explanation is that the Box 1 regions of these receptors interact with specific tyrosine kinases that transmit growth proliferative signals and, in the case of hG-CSFR, a kinase responsible for tyrosine phosphorylation of p75<sup>corel</sup>.

Requirement of the Box 1 and Box 2 motifs for mitogenic signaling and tyrosine phosphorylation of Jak2 has been reported for EPO-R, GM-CSFR, and IL-3R. An EPO-R mutant containing a point mutation at the conserved W residues within the conserved Box 1 motif of hG-CSFR and gp130 with serine was shown to result in the loss of signal-transducing capacity by gp130 and an associated disappearance of tyrosine phosphorylation of gp130 and Jak2. The mechanism by which substitution of these two critical proline residues alone in hG-CSFR and gp130 results in inactivation of these receptors for mitogenic activity and protein tyrosine phosphorylation is unknown at present. It is possible that substitution of the two proline residues changes the tertiary structures of the cytoplasmic regions of hG-CSFR and gp130, as proline is a strong helix breaker and believed to be important in protein structure. An alternative explanation is that the Box 1 regions of these receptors interact with specific tyrosine kinases that transmit growth proliferative signals and, in the case of hG-CSFR, a kinase responsible for tyrosine phosphorylation of p75<sup>corel</sup>.

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Fig 7. Identification of p75 as c-rel. Ba/F3 cells expressing the D7 wild-type hG-CSFR splice variant were unstimulated (−) or stimulated (+) with 10 ng/mL G-CSF for 10 minutes at 37°C. (A) Cell lysates were immunoprecipitated with c-rel antibody before electrophoresis and then analyzed by immunoblotting with 4G10 antibody. (B) Cell lysates were immunoprecipitated with 4G10 antibody, electrophoresed, and immunoblotted with c-rel antibody.

Fig 8. The major component of the 75-kDa tyrosine-phosphorylated band is c-rel. Ba/F3 cells expressing the D7 WT hG-CSFR were unstimulated (−) or stimulated (+) with 10 ng/mL G-CSF for 10 minutes at 37°C. (A) Cell lysates were immunoprecipitated (IP) with c-rel antibody (α c-rel), electrophoresed, and immunoblotted (Blot) with 4G10 antibody (α 4G10). (B) The unbound (pre-cleared with α c-rel) fraction from (A) was immunoprecipitated with α 4G10, electrophoresed, and immunoblotted with α 4G10. Arrows indicate migration of p75<sup>corel</sup>.
amino acids of the murine G-CSFR are required for G-CSF-dependent proliferation in FDC-P1 cells. Within the critical 53-amino acid cytoplasmic signal transducing region of hG-CSFR lie the Box 1 and Box 2 sequence motifs, which are conserved among members of the cytokine receptor superfamily. Termination of hG-CSFR between these two motifs has been reported to inactivate the receptor for growth signal transduction. Truncations or point mutations in either of these two conserved sequence motifs in the signal-transducing gp130 molecule completely abolish the IL-6-mediated growth signal. Mutations in the Box 2 region have also been shown to significantly affect or inactivate the function of EPO-R and IL-2Rβ chain, respectively. Collectively, these observations suggest that Box 2 is the most distal region that is required for cytokine receptor growth signaling.

Our data with the mitogenically impaired Cfr mutant suggest that additional downstream sequences are required for delivery of a full proliferative signal and, perhaps, for interaction with c-rel. Other studies have emphasized the significance of residues distal to Box 2 of hG-CSFR in augmenting its mitogenic potential and in inducing acute-phase protein gene expression in hepatoma cell line transfectants. Several recent studies have also indicated the importance of amino acids distal to Box 2 within the so-called extended Box 2 subdomain of EPO-R for efficient mitogenesis. Presumably, a domain for unknown enzymatic activity or for interaction with other cytoplasmic molecules is located within close proximity of Box 2 of the cytosolic portion of hG-CSFR and EPO-R.

We do not yet know which signal transduction pathways are uniquely activated in induction of a proliferative versus maturation response by hG-CSFR. The C-terminal region of the cytoplasmic domain of the G-CSFR has recently been shown to be essential for induction of myeloperoxidase gene expression and transduction of maturation signals. Further studies to determine the specific tyrosine kinases activated and downstream signaling molecules will be required to dissect signal transduction pathways used by hG-CSFR to transduce proliferative versus maturation signals.

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Point mutations in the conserved box 1 region inactivate the human granulocyte colony-stimulating factor receptor for growth signal transduction and tyrosine phosphorylation of p75c-rel

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