Divergent Pathways in COS-7 Cells Mediate Defective Internalization and Intracellular Routing of Truncated G-CSFR Forms in SCN/AML

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Abstract

Background: Expression of truncated G-CSF forms in patients with SCN/AML induces hyperproliferation and prolonged cell survival. Previously, we showed that ligand internalization is delayed and degradation of truncated G-CSFR forms is defective in patients with SCN/AML.

Methodology/Principal Findings: In this study, we investigated the potential roles of dileucine and tyrosine-based motifs within the cytoplasmic domain of the G-CSFR in modulating ligand/receptor internalization. Using standard binding assays with radiolabeled ligand and COS-7 cells, substitutions in the dileucine motif or deletion of tyrosine residues in the G-CSFR did not alter internalization. Attachment of the transferrin receptor YTRF internalization motif to a truncated G-CSFR form with a patient with SCN/AML corrected defective internalization, but not receptor degradation suggesting that receptor internalization and degradation occur independently via distinct domains and/or processes.

Conclusions: Our data suggest that distinct domains within the G-CSFR mediate separate processes for receptor internalization and degradation. Our findings using standard binding assays differ from recently published data utilizing flow cytometry.

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Introduction

Granulocyte colony-stimulating factor (G-CSF) critically regulates neutrophil numbers by binding to the G-CSF receptor (G-CSFR) to generate signals that maintain a homeostatic balance between myeloid cell survival, proliferation, and differentiation [1–4]. Despite continued progress in our understanding of the signaling pathways that are activated by the G-CSFR to transduce mitogenic signals, little is currently known about the mechanisms by which the G-CSFR downregulates proliferative signaling.

For those growth factor receptors that have been studied, ligand-induced receptor internalization has been shown to critically control cellular responsiveness [5–9]. Following ligand binding, most receptors are recruited to clathrin-coated pits where they are endocytosed then internalized and either recycled back to the cell surface or degraded within intracellular compartments. Sorting signals within the cytosolic tails of the receptors have been identified that direct trafficking of the receptors [10]. Tyrosine-containing and dileucine-containing regions are two characteristic motifs responsible for endosomal-lysosomal targeting of receptor proteins. Receptor internalization and degradation serve to attenuate receptor signaling by rapidly down-modulating or decreasing the number of receptors on the cell surface to protect cells from over-stimulation. Receptor trafficking also controls the specific signaling pathways triggered by activated receptors and the intensity of signaling [9,11,12].

Abnormalities in both receptor oligomerization and trafficking have been shown to alter normal receptor function and ligand sensitivity and to contribute to human diseases [13–16]. Our laboratory previously reported that ligand internalization and receptor degradation are impaired leading to sustained cellular activation and enhanced cell survival and proliferation in patients with severe congenital neutropenia (SCN) transforming to AML in which a truncated G-CSFR is expressed along with the wild type form [17]. Our data were subsequently confirmed by others in both in vitro and in vivo studies [18,19].

The precise defects responsible for abnormal degradation and modulation of surface expression of the G-CSFR in SCN/AML remain unknown. In this study, we have examined the roles of known internalization motifs within the cytoplasmic domain of the...
G-CSFR to determine whether they modulate G-CSFR internalization and degradation. Our data suggest that the G-CSFR utilizes distinct and separate processes for receptor internalization and degradation as a mechanism for regulating receptor surface expression and recruitment of specific signaling pathways.

**Methods**

**Reagents**

Cell culture reagents, restriction enzymes, and oligonucleotides were purchased from Invitrogen Inc. (Carlsbad, CA). G-CSF was a generous gift from Amgen Inc (Thousand Oaks, CA). [125I]G-CSF (>800Ci/mmol) and Pro-mix [35S] in vivo cell labeling mix (>1000Ci/mmol) were obtained from Amersham (Arlington Heights, IL). All other reagents were purchased from Sigma Chemical Co (St. Louis, MO) unless otherwise indicated.

**DNA constructs**

The construction of pCDNA3.1-WT, the wild-type human Class I G-CSFR cDNA (generously provided by Dr. A. Larsen, Seattle, WA) and pCDNA3.1-ΔT16 have been previously reported [17]. Tyrosine to phenylalanine (Y→F) mutations at amino acid positions 729 and 764 of the WT G-CSFR were introduced by site directed mutagenesis and the corresponding G-CSFR forms were designated Y729F and Y764F, respectively. To generate Y729F and Y764F, an A to T point mutation at nt. 2465 or 2525 was introduced, respectively, in the WT-G-CSFR cDNA by overlap extension PCR [17]. The oligonucleotides used to generate these mutants were: forward primer Y729F F1 (5’-GGACCGGACGCTGTCACAGG-3’), forward primer Y764F F2 (5’-GGCCCAAAATGTGCAGCG-3’), reverse primer Y729F R1 (5’-GCCGGAACAAACACTCTTCAGG-3’), reverse primer Y729R (5’-GGACCGGACGCTGTCACAGG-3’); the underlined nucleotides indicate the positions of the point mutations. To generate Y744F, primer F3 containing a 5’ restriction site for BamHI and corresponding to nt. 2252-2268 of the WT-G-CSFR cDNA was used in conjunction with the Y729F R1, to amplify WT-G-CSFR cDNA. In a separate PCR reaction, WT-G-CSFR cDNA was amplified with Y729F F1 primer and primer R3 which was designed to contain a XhoI restriction site and corresponded to nts. 2581-2596 of the WT-G-CSFR cDNA. The products from the two initial PCR reactions were combined and amplified by PCR using the external primers F3 and R3. The PCR product was digested with XhoI and BamHI and then subcloned into the XhoI and BamHI sites of pBluescript SK+. An internal 2680bp fragment in the cloned product was excised from pBluescript SK+ by Cfr101 and BstEII digestion, gel purified, and cloned into WT cDNA replacing WT G-CSFR to determine whether they modulate G-CSFR internalization and degradation. Our data suggest that the G-CSFR utilizes distinct and separate processes for receptor internalization and degradation as a mechanism for regulating receptor surface expression and recruitment of specific signaling pathways.

To generate the Y764F mutant, primer pairs Y764F F2/R3 and Y764F R2 (5’-GGCCCAAAATGTGCAGCG-3’, reverse primer Y764F R2 containing a 5’ restriction site for XhoI and a 3’ restriction site for BamHI) were used in initial PCR reactions and the resulting PCR products further amplified with oligonucleotides F3 and R3. The same cloning strategy was used to generate pCDNA3.1-Y729F.

**Internalization studies**

Ligand internalization was performed as previously reported [17]. Briefly, cells were incubated with 500pM [125I]G-CSF for 2 h at 4°C. Internalization of radiolabeled ligand was examined by temperature shifting to 37°C for varying times from 30 to 120 mins. Following incubation at 37°C, the cells were incubated for an additional 2 h at 4°C. To remove unbound ligand, cells were washed in cold PBS containing 1mM MgCl2, 0.1mM CaCl2, and 0.2% BSA. Surface-bound [125I]G-CSF was removed by incubation with 0.5M NaCl (pH1.0) for 3 mins followed by washing in cold PBS solution. The acid strip solution and wash were both collected to determine bound ligand. Internalized ligand was quantified by lysis of the cells with 1m NaOH for 1min. The data were expressed as percent internalization over time [20]. Analysis of specific binding was also performed in the presence of excess cold ligand as previously described [17,21,22].

**Receptor degradation**

Transfected COS-7 cells grown to confluence in T75 flasks were incubated with short-term labeling media (RPMI 1640 media...
containing 10% dialyzed FBS without methionine and cysteine) for 15 mins at 37°C. Cells were metabolically labeled with [35S] Cysteine/Methionine Pro-mix at 0.15mCi/ml in short-term labeling media for 1 h at 37°C as previously described [10,20,23]. The cells were washed with incubation media (RPMI 1640 containing 10% FBS, 1nM G-CSF, and unlabeled methionine and cysteine) and incubated for varying times, then washed once in PBS, scraped and pelleted. The cell pellets were lysed in buffer containing 1% NP-40 (Boehringer Mannheim Biochemical, Indianapolis, IN), 1mM EDTA (pH 8.0), 20mM Tris lysed in buffer containing 1% NP-40 (Boehringer Mannheim methionine and cysteine) and incubated for varying times, then washed once in PBS, scraped and pelleted. The cell pellets were lysed in buffer containing 1% NP-40 (Boehringer Mannheim Biochemical, Indianapolis, IN), 1mM EDTA (pH 8.0), 20mM Tris (pH 8.0), 150mM NaCl, 0.15U/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A and 1mM sodium vanadate, then cleared of insoluble material. Lysates were pre-cleared with protein A agarose (Invitrogen), immunoprecipitated with 8 µg anti-G-CSFR antibody recognizing the N-terminal portion of the G-CSFR (BD PharMingen, San Diego, CA), and analyzed under reducing conditions by SDS-PAGE. The gels were treated with Entensify (DuPont-NEN, Wilmington, DE) according to the manufacturer’s instructions and dried. The labeled proteins were visualized by autoradiography.

Results

Role of dileucine and tyrosine-based motifs in the G-CSFR in ligand-receptor internalization

Since efficient internalization of many growth factor receptors has been shown to be mediated by dileucine or tyrosine-based motifs in the cytoplasmic domains of these receptors [24–26], we investigated whether dileucine and tyrosine-based motifs present in the WT G-CSFR but absent in the Δ716 G-CSFR form might mediate internalization of the G-CSFR complex. Mutations in the G-CSFR corresponding to these motifs were therefore introduced.

In previous studies using the Ba/F3 pro-B cell line, and COS-7 cells expressing the WT and truncated G-CSF forms, we demonstrated that ligand internalization and down-modulation of G-CSFR expression were impaired in cell lines expressing the Δ716 G-CSFR, which is the most common mutant G-CSFR form identified in patients with AML and antecedent SCN [17]. The Δ716 G-CSFR form arises due to a C to T substitution resulting in the generation of a premature stop codon (Figure 1).

To confirm whether our observations of defective internalization and prolonged receptor surface expression in transfected Ba/F3 and COS-7 cells held true in myeloid cells, studies with primary myeloid cells from mice expressing the Δ716 G-CSFR or the truncated form of the G-CSFR were done. Similar to our in vitro studies, we observed that G-CSF internalization and down-modulation of the G-CSFR in myeloid cells from the “knock-in” mice are also impaired (data not shown). Since these observations were consistent with our previous in vitro observations [17], additional studies were performed using COS-7 cells.

The WT G-CSFR contains an STQPLL dileucine motif in its cytoplasmic domain at aa. 749–754 which is identical to the dileucine motif in the cytoplasmic domain of the homologous gp130 molecule which mediates IL-6 internalization and down-modulation of the IL-6R [20,23]. Both leucine residues in the dileucine motif of gp130 have been shown to be essential for IL-6 internalization [27]. In temperature-shift and acid washing studies with G-CSFR mutants containing either single or double leucine to alanine substitutions at residues 753 and 754, no significant differences in internalized ligand were observed between the WT and L→A transfectants (Figure 2). Ligand internalization was apparent within 30 minutes in cells expressing L753A, L754A, L753/754A, or the WT G-CSFR, with nearly 40% of bound ligand internalized by 30 minutes. Only small increases in internalized ligand were observed at 1 h and 2 h, suggesting ligand internalization is nearly complete by 30 minutes. In contrast, ligand internalization was significantly delayed in the truncated Δ716 G-CSFR (p<0.05 for 30-120 minute time points, compared to WT). These results suggest that unlike the homologous gp130 molecule other cytoplasmic motifs in the G-CSFR mediate ligand/receptor internalization.

Tyrosine-based motifs in the G-CSFR do not mediate ligand-receptor internalization

We next investigated the role of tyrosine residues in the cytoplasmic tail of the G-CSFR in mediating receptor internalization. Since Tyr729 and Tyr764 correspond to known tyrosine-based motifs for other receptors [28] and are deleted in the Δ716 G-CSFR, G-CSFR mutants containing tyrosine to phenylalanine mutations at these locations were generated and designated Y729F and Y764F, respectively. As shown in Figure 3, significant levels of internalized [125I]G-CSF were detected in cells expressing the Y729F and Y764F G-CSFR forms. There was no evidence for defective ligand internalization with the Y729F or Y764F G-CSFR forms compared to the WT G-CSFR over the 2 h time period examined. Although the Y729F receptor mutant exhibited a trend suggesting accelerated internalization compared to the WT receptor form, this was found to be not statistically significant (p = 0.37). These results suggest that loss of either tyrosine residue at position 729 or 764 does not alter ligand/receptor internalization. We also examined the remaining two G-CSFR cytoplasmic tyrosine residues at positions 704 and 744 by introducing tyrosine to phenylalanine mutations and examining ligand internalization in COS-7 cells transfected with these mutant G-CSFR forms, and observed no appreciable alterations in ligand/receptor internalization (data not shown).

Effect of a C-terminal YTRF internalization motif on internalization and degradation of the Δ716 G-CSFR

Since our collective data indicated that other sequences in the G-CSFR must regulate ligand/receptor internalization, and since we previously showed that both internalization and degradation of ligand/receptor complexes are impaired in cells expressing the Δ716 G-CSFR, we were interested in determining whether insertion of a known internalization motif into the Δ716 G-CSFR could correct these defects. For these studies, the YTRF internalization motif of the transferrin receptor [23] was inserted in-frame and 5’ to the stop codon of the truncated Δ716 G-CSFR and ligand/receptor internalization and degradation were examined. As shown in Figure 4A, attachment of the YTRF motif corrects the defect in ligand/receptor internalization in cells expressing the Δ716-YTRF G-CSFR. Internalized 125I-labeled G-CSF was observed within 30 minutes in cells expressing the WT G-CSFR or the Δ716-YTRF G-CSFR to which the YTRF motif had been attached. At each time point examined, cells expressing the Δ716-YTRF receptor form had significantly higher levels of internalized ligand compared to the Δ716 G-CSFR lacking the fused internalization motif (p<0.01 at each time point).

We next examined whether correction of the internalization defect in Δ716 cells by attachment of the YTRF motif also restored receptor degradation back to normal. We have previously shown that degradation of the WT G-CSFR occurs within 2 h following ligand exposure [17], whereas degradation of the truncated Δ716 G-CSFR is delayed even at 4 h. As shown in Figure 4B, there was no evidence that attachment of the YTRF motif corrected the defect in receptor degradation in cells expressing the Δ716 G-CSFR. The half-life of the WT G-CSFR

![Image](image-url)
was determined to be 3.62 ± 0.15 compared to 4.84 ± 0.11 and 5.9 ± 1.02 for Δ716 and Δ716-YTRF, respectively (WT vs. Δ716, p = 0.013 and WT vs. Δ716-YTRF, p = 0.13). No significant difference in receptor half-life was observed between Δ716 and Δ716-YTRF G-CSFR forms (p = 0.24).

Discussion

SCN is characterized by neutropenia with frequent and often severe bacterial infections [29]. The neutropenia of these patients is generally responsive to pharmacological doses of G-CSF. However, 21% of patients will ultimately develop AML [30]. Mutations in one allele of the gene for the G-CSFR have been identified in 78% of these patients [30,31]. These mutations introduce a premature stop codon that truncates the cytoplasmic tail of the G-CSFR resulting in hypersensitivity to G-CSF, resistance to apoptosis, and enhanced cell proliferation. Collectively, these observations have led to the postulation that G-CSFR mutations contribute to leukemogenesis in patients with SCN [32].

We and others have previously demonstrated sustained intracellular signaling in response to G-CSF in cells expressing truncated G-CSFR forms isolated from patients with SCN/AML [18,19,33]. Our laboratory has also shown that internalization and degradation of the truncated G-CSFR forms is impaired [17].

Figure 1. Schematic diagram of G-CSFR forms. The extracellular (EX), transmembrane (TM), and intracellular (ID) domains of the various G-CSFR forms are shown with the conserved box 1, 2, and 3 regions indicated. The full-length wild-type (WT) G-CSFR contains four cytoplasmic tyrosine residues at a.a. positions 704, 729, 744, and 764. The location of the dileucine motif in the WT G-CSFR at residues 749-754, which is deleted in Δ716, where Leu to Ala substitutions were introduced to generate the L753A, L754A, and the L753/754A G-CSFR is shown. Tyr to Phe substitutions at either Tyr729, Tyr744, or Tyr764 were also introduced and the corresponding G-CSFR mutants designated Y729F, Y744F, and Y764F, respectively. The Δ716 G-CSFR was isolated from a patient with SCN/AML and contains a premature stop codon resulting in a truncated G-CSFR. The Δ716-YTRF mutant was generated by attachment of the transferrin receptor YTRF internalization motif S’ to the stop codon of the Δ716 G-CSFR.

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Thus similar to other human disorders such as Laron dwarfism, type A insulin resistance, and familial hypercholesterolemia in which aberrant down-modulation of growth factor receptors has been shown to play a role in the pathophysiology of these diseases [13–16], disruption of receptor trafficking also appears to underlie altered cell signaling in SCN/AML. In this study, we have further investigated the mechanisms that modulate G-CSFR expression and are responsible for G-CSFR internalization and degradation.

For other growth factor receptors, down-modulation of their surface expression has been shown to be mediated by specific amino acid motifs in the receptors themselves and/or involve the recruitment and association of adaptor proteins with the receptors which then target the receptors to intracellular compartments for degradation, recycling, or both [28]. Dileucine motifs in a number of cell surface proteins have been reported to mediate their internalization and sorting in the trans-Golgi apparatus as well as trafficking to the lysosome [24,26]. Leucine-based internalization motifs have been identified in the mannose 6-phosphate/Insulin – like growth factor receptor, insulin receptor, CD3γ chain, epidermal growth factor receptor, leukemia inhibitory factor

**Figure 2. Leucine residues in the STPQLL dileucine motif do not mediate G-CSFR internalization.** Mutations in the cytoplasmic dileucine motif of the G-CSFR that is identical to gp130 were introduced by substituting Ala for either Leu753 (L753A) or Leu754 (L754A) or both Leu753 and Leu754 (L753/754A). Binding and internalization of [125I]-G-CSF were analyzed in COS-7 cells transfected with the WT G-CSFR, Δ716, or G-CSFR forms with Leu to Ala mutations in the dileucine motif. Average data ± S.E.M (n = 4) are shown and are expressed as the percent of initial binding at time 0 at 4°C. doi:10.1371/journal.pone.0002452.g002

**Figure 3. Binding and internalization of [125I]-G-CSF in cells transfected with WT, Y729F or Y764F G-CSFR forms.** Mutations in the G-CSFR were introduced by substitution of Phe for either Tyr729 (Y729F) or Tyr764 (Y764F). COS-7 cells were transfected with each G-CSFR form and ligand binding analyzed. Surface bound ligand was quantitated by acid stripping in 0.5M NaCl (pH 1.0). Internalized ligand was measured after lysis of the cells in 1M NaOH. Data are expressed as a percentage of initial binding at time 0 at 4°C. Values represent the average ± S.E.M (n = 2). doi:10.1371/journal.pone.0002452.g003
receptor, and gp130 subunit of the IL-6 receptor complex [7,9,20,23,25,26,34,35]. In the gp130 subunit, substitution of the leucine residues in the dileucine STQPLL motif with alanines was shown to dramatically alter trafficking of the IL-6 receptor complex with a near complete loss in the capacity to internalize the receptor complex [20]. In addition, tyrosine containing motifs have also been identified that mediate the internalization of several growth factor receptors, including the receptors for transferrin, low density lipoprotein, IL-2-receptor β chain, and receptors for the insulin-like growth factor (IGF) and mannose 6-phosphate as well as the B-cell receptor α chain,[28,36–40].

Since the identical STQPLL dileucine motif in gp130 that mediates IL-6R internalization is also present at residues 749–754 in the G-CSFR [17,20,23], we were interested in determining the role of this motif in internalization of the G-CSFR. They reported that mutation of both leucines or the serine residue in the cytoplasmic dileucine motif of the G-CSFR resulted in approximately 50% reduction in receptor endocytosis in 32D cells [41]. The reasons for the discrepancies in our findings and theirs are not clear. It is possible that the differences relate to differences in the cell models used. Since a potential kinase that binds to the dileucine motif to mediate G-CSFR internalization has been postulated [41], it is possible that the unknown kinase is not expressed in COS-7 fibroblast cells. Previous studies with the homologous gp130 molecule of the IL-6R demonstrating a role for the STQPLL motif in receptor internalization utilized a fibroblast model [20,23]. Hence unlike

![Figure 4. Effect of attachment of an YTRF internalization motif to the Δ716 G-CSFR on ligand/receptor internalization and degradation.](image-url)

(A) Cells expressing the WT, Δ716, or Δ716-YTRF G-CSFR forms were incubated with [125I]-labeled G-CSF and subjected to temperature shift and acid washing to determine surface-bound and internalized ligand. Average data ± S.E.M (n = 4) are shown as a percentage of initial binding of G-CSF at time 0 at 4°C. (B) Cells expressing the WT, Δ716 or Δ716-YTRF G-CSFR forms were metabolically labeled with [35S]-methionine and [35S]-cysteine for 1 h, washed then incubated in media containing 1 nM G-CSF and unlabeled amino acids at 37°C. At the indicated times, the cells were lysed, immunoprecipitated with antibody recognizing the N-terminus of the human G-CSFR and analyzed by SDS-PAGE. The blots were subjected to densitometry and the average pixels ± S.E.M are shown (n = 2). A representative blot from two independent experiments is shown.

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