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Multiple Signals Mediate Proliferation, Differentiation, and Survival from the Granulocyte Colony-stimulating Factor Receptor in Myeloid 32D Cells*

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Granulocyte colony-stimulating factor (G-CSF) regulates neutrophil production through activation of its cognate receptor, the G-CSF-R. Previous studies with deletion mutants have shown that the membrane-proximal cytoplasmic domain of the receptor is sufficient for mitogenic signaling, whereas the membrane-distal domain is required for differentiation signaling. However, the function of the four cytoplasmic tyrosines of the G-CSF-R in the control of proliferation, differentiation, and survival has remained unclear. Here we investigated the role of these tyrosines by expressing a tyrosine "null" mutant and single tyrosine "add back" mutants in maturation-competent myeloid 32D cells. Clones expressing the null mutant showed only minimal proliferation and differentiation, with survival also reduced at low G-CSF concentrations. Analysis of clones expressing the add-back mutants revealed that multiple tyrosines contribute to proliferation, differentiation, and survival signals from the G-CSF-R. Analysis of signaling pathways downstream of these tyrosines suggested a positive role for STAT3 activation in both differentiation and survival signaling, whereas SHP-2, Grb2 and Shc appear important for proliferation signaling. In addition, we show that a tyrosine-independent "differentiation domain" in the membrane-distal region of the G-CSF-R appears necessary but not sufficient for mediating neutrophilic differentiation in these cells.

The production of blood cells is regulated by a range of extracellular stimuli, including a network of hematopoietic growth factors and cytokines. One of these, granulocyte colony-stimulating factor (G-CSF),[1] is a major regulator of neutrophilic granulocyte production and augments the proliferation, survival, maturation, and functional activation of cells of the granulocytic lineage (1–4). The actions of G-CSF are mediated through its interaction with a specific cell surface receptor, the G-CSF-R, which forms homo-oligomeric complexes upon ligand binding (5). Typical of other members of the hematopoietin receptor superfamily, the G-CSF-R has no intrinsic tyrosine kinase activity but activates cytoplasmic tyrosine kinases (2, 5, 6). Important signaling molecules utilized by the G-CSF-R include the Janus tyrosine kinases Jak1, Jak2, and Tyk2 (7–10), the Src kinases $p55^{lyn}$ and $p56/59^{hck}$ (11–13), the signal transducer and activator of transcription (STAT) proteins STAT1, STAT3, and STAT5 (9, 14–19), and components of the $p21^{ras}$/Raf/mitogen-activated protein kinase pathway (8, 20–24).

The cytoplasmic region of the G-CSF-R can be subdivided into a membrane-proximal domain, which contains two conserved subdomains known as box 1 and box 2, and a membrane-distal domain, which contains a less-conserved box 3 sequence (5). In myeloid cells, the membrane-proximal domain is essential for mitogenic signaling, whereas the membrane-distal domain is essential for the transduction of differentiation signals (19, 25–27). In addition, there are four tyrosine (Tyr) residues in the cytoplasmic region of the G-CSF-R, at positions 704, 729, 744, and 764 of the human receptor, three of which lie in the membrane-distal domain (28). Ligation of the G-CSF-R results in the rapid phosphorylation of these four tyrosines (7, 29), which form potential binding sites for signaling molecules that contain Src homology 2 (SH2) or phosphotyrosine binding domains (30, 31). Some signaling pathways emanating from the different tyrosines of the G-CSF-R have been identified. For example, we and others have shown that Tyr-704 and Tyr-744 of the G-CSF-R are involved in the recruitment and activation of STAT3 by the G-CSF-R (15, 17, 18, 32). In addition, Tyr-764 is necessary for the formation of Shc/Grb2/p140 complexes as well as the activation of $p21^{ras}$ (22, 33). However, studies to determine the role of each of the four cytoplasmic tyrosines in mediating the effects of G-CSF on proliferation, differentiation, and survival have yielded ambiguous and somewhat conflicting results (15, 17, 21, 22, 29, 33, 34).

To better define the role of receptor tyrosines in signaling from the G-CSF-R, we examined the ability of a tyrosine null mutant and a series of single tyrosine add back mutants to transduce biological signals in response to G-CSF. Furthermore, we performed these studies in maturation-competent myeloid 32D cells, which are able to closely recapitulate many of the cellular responses to G-CSF, including proliferation, survival, and, importantly, terminal differentiation into mature neutrophils (27). Thus, 32D cells provide an appropriate cellular context to assess the physiological relevance of signals from the G-CSF-R. This analysis revealed that multiple tyrosines contribute to proliferation, differentiation, and survival signaling from the human G-CSF-R. Analysis of signaling pathways downstream from these tyrosines suggest a positive role for STAT3 activation in both differentiation and survival,

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[1] The abbreviations used are: G-CSF-R, granulocyte colony-stimulating factor receptor; STAT, signal transducer and activator of transcription; SH2, Src homology 2; WT, wild type; IL-3, interleukin 3; TBST, Tris-buffered saline-Tween; GAP, GTPase-activating protein.

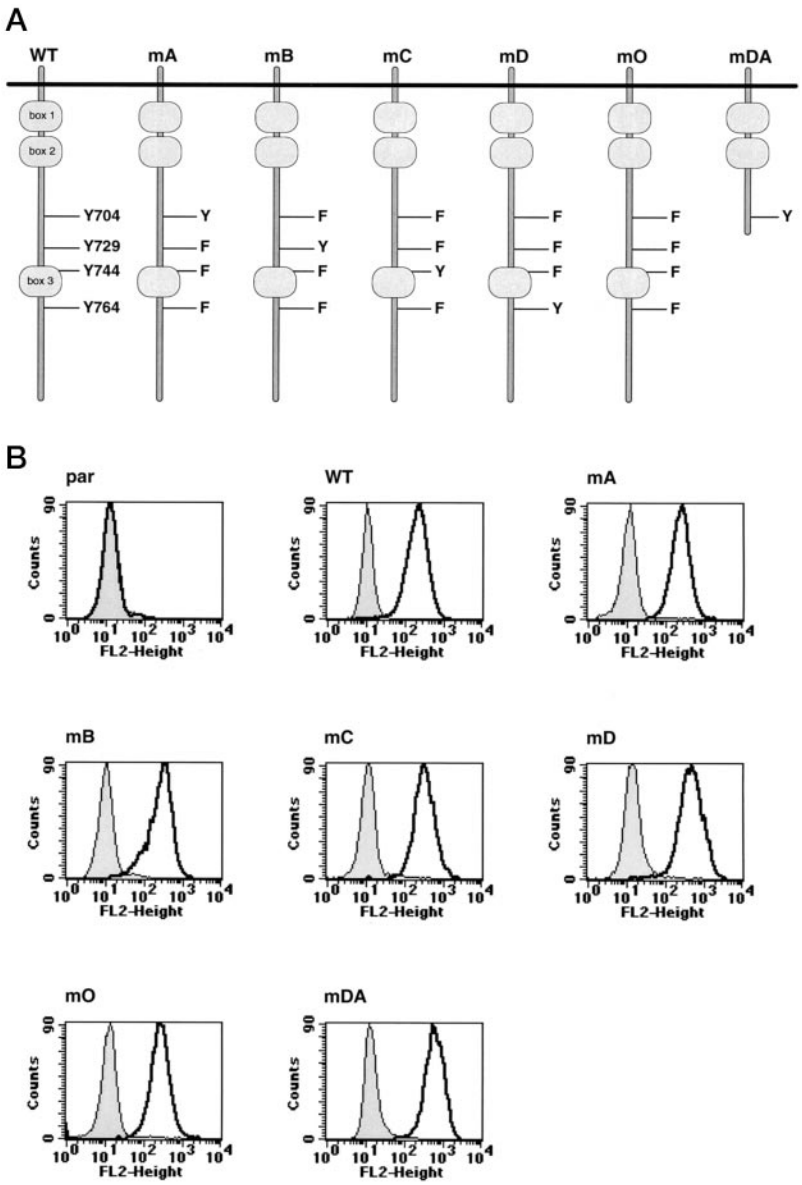

FIG. 1. **Expression of G-CSF-R mutants in myeloid 32D cells.** *A*, schematic diagram of the cytoplasmic domains of mutant G-CSF-R proteins. *Boxes 1*, *2*, and *3* denote subdomains conserved in several members of the hematopoietin receptor superfamily (5). *B*, flow cytometric analysis of G-CSF-R expression on parental 32D.cl8.6 cells (par) or transfectants. Cells were either stained with biotinylated mouse anti-human G-CSF-R antibodies followed by phycoerythrin-conjugated streptavidin, biotinylated anti-streptavidin, and finally phycoerythrin-conjugated streptavidin (*unfilled*) or without the anti-G-CSF-R step (*filled*) and analyzed for fluorescence (*FL2-Height*).

whereas SHP-2, Grb2, and Shc appear important for proliferation. In addition, we show that a tyrosine-independent "differentiation domain" in the membrane-distal region of the G-CSF-R appears necessary, although not sufficient, for mediating neutrophilic differentiation in 32D cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection of G-CSF-R Constructs—The pLNCX expression constructs of human G-CSF-R wild-type (WT), a series of triple Tyr → Phe add back mutants (mA, mB, mC, and mD), a quadruple Tyr → Phe null mutant (mO), and a truncation mutant derived from a patient with severe congenital neutropenia (mDA) have been described previously (18, 27). A subline of the IL-3-dependent murine myeloid cell line 32D.cl3, called 32D.cl8.6, which lacked endogenous G-CSF-R expression but remained maturation-competent, was generated as described previously (27). This was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 10 ng/ml murine IL-3. The expression constructs were linearized by *Pvu*I digestion and transfected into 32D.cl8.6 cells by electroporation. After 48 h of incubation, cells were selected with G418 (Life Technologies, Inc.) at a concentration of 0.8 mg/ml. Multiple clones were expanded for further analysis. To check G-CSF-R expression levels, cells were incubated at room temperature for 60 min with 10 μg/ml biotinylated mouse anti-human G-CSF-R monoclonal antibody LMM741 (PharMingen, San Diego, CA), then at 4 °C for 60 min sequentially with 5 μg/ml phycoerythrin-conjugated streptavidin, 5 μg/ml biotinylated anti-streptavidin antibody, and finally 2 μg/ml phycoerythrin-conjugated streptavidin, with washing between each antibody step. Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Several independently derived cell lines of each construct were selected on the basis of equivalent receptor expression.

Cell Proliferation and Morphological Analysis—To determine the proliferation and differentiation characteristics of 32D.cl8.6 clones, cells were incubated at an initial density of 1–2 $\times$ 10^5 cells/ml in RPMI medium supplemented with 10% fetal calf serum and either 100 ng/ml human G-CSF, 10 ng/ml of murine IL-3, or without growth factors. The medium was replenished every 1–2 days, and the cell densities were adjusted to 1–2 $\times$ 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 by May-Grünwald-Giemsa staining. To quantify the neutrophilic maturation of 32D.cl8.6 transfectants in response to G-CSF, the number of terminally differentiated cells was determined and expressed as a percentage of total living cells (% neutrophils).

Preparation of Cell Lysates and Western Blotting—Cells were deprived of serum and factors for 4 h at 37 °C in RPMI 1640 medium at a density of 1 $\times$ 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 volumes of ice-cold phosphate-buffered saline supplemented with 10 μM Na_3VO_4 were added. Subsequently, cells were pelleted and lysed by incubation for 30 min at 4 °C in Tyr(P) lysis buffer (1% Triton X-100, 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1 mM

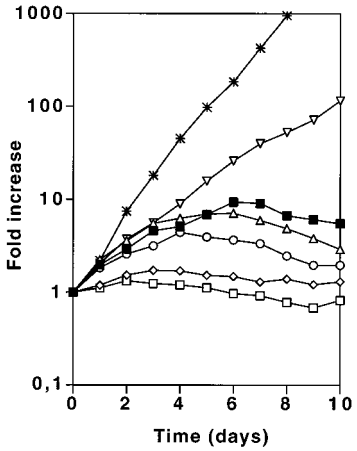

FIG. 2. **Proliferation of 32D clones in response to G-CSF.** Cell-proliferation of 32D.cl8.6 clones expressing WT or mutant G-CSF-Rs, as indicated, in response to 100 ng/ml G-CSF. Data represent the mean growth of three independent clones for each receptor construct. ■, WT; △, mA; ◇, mB; ○; mC; ▽, mD; □, mO; ✷, mDA.

Na_3VO_4, 1 mM dithiothreitol, 1 mM Pefabloc SC, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 50 μg/ml bacitracin) followed by centrifugation at 13,000 × *g* for 15 min. The soluble proteins were mixed with sample buffer, separated by SDS-polyacrylamide gel electrophoresis (SDS-polyacrylamide gel electrophoresis), and transferred onto nitrocellulose (0.2 μm; Schleicher & Schuell). Filters were blocked by incubation in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20) containing 0.6% (w/v) bovine serum albumin. Antibodies used for Western blotting were anti-phospho-STAT3[Y705] (New England Biolabs, Inc. Beverly, MA), anti-phospho-STAT3[S727] (New England Biolabs), and anti-STAT3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and were diluted in TBST containing 0.6% (w/v) bovine serum albumin. After washing with TBST, immune complexes were detected with horseradish peroxidase-conjugated species-specific antiserum (DAKO, Glostrup, Denmark) followed by enhanced chemiluminescence reaction (DuPont). In some instances, membranes were stripped in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol at 50 °C for 30 min, reblocked, washed, and reprobed.

Far Western Analysis—The cytoplasmic domain of the human G-CSF-R was cloned into pET-15b (Novagen, Madison, WI) as described (18). In addition, the following glutathione *S*-transferase fusion constructs were made utilizing standard polymerase chain reaction protocols to amplify the appropriate coding regions, which were cloned into pGEX-2T (Amersham Pharmacia Biotech): Grb2-FL (SH3-SH2-SH3), Grb2-FL(mut) (SH3-SH2mutant-SH3), Shc-SH2, SHP-1-SH2(N), SHP-1-SH2(C), SHP-2-SH2(N), SHP-2-SH2(C), Syk-SH2(N), and Syk-SH2(C). The authenticity of all constructs was verified by DNA sequencing. In addition, constructs were obtained for the production of glutathione *S*-transferase fusions with GAP-SH2(N) and GAP-SH2(C) from Tony Pawson, Vav-SH2 and Fps-SH2 from Lewis Cantley, CrkL-SH2 and Abl-SH2 from Wallace Langdon, and Grb14-SH2 from Roger Daly. For the production of tyrosine-phosphorylated G-CSF-R cytoplasmic domain, the pET clone was introduced into the *E. coli* strain TKB1 (Stratagene, La Jolla, CA), which contains an inducible tyrosine kinase. Fusion protein was produced and purified according to the manufacturer's instructions, then ^{32}P-labeled using heart muscle kinase. For the production of glutathione *S*-transferase fusions, plasmids were transformed into XL-1 Blue (Stratagene), with proteins expressed and purified on glutathione-Sepharose 4B beads as described (35). These proteins were then electrophoresed on 10% SDS-polyacrylamide gel electrophoresis gels (36) and electrophoretically transferred to Hybond-C membranes (Amersham Pharmacia Biotech). The membranes were processed through a denaturation-renaturation cycle (37) and probed with the ^{32}P-labeled G-CSF-R, as described (38).

RESULTS

Expression of G-CSF-R Mutants in Maturation-competent 32D Cells—The contribution of receptor tyrosines to G-CSF-R function has remained unclear (15, 17, 21, 22, 29, 33, 34). Therefore, to better understand the role of these tyrosines, we analyzed a quadruple Tyr → Phe or null mutant with no cytoplasmic tyrosines (mO) and a series of triple Tyr → Phe or add back mutants, which each retain a single cytoplasmic tyrosine (mA, mB, mC, and mD). Expression vectors encoding these mutant receptors along with WT and truncated (mDA) G-CSF-Rs (Fig. 1*A*) were introduced into a subline of the IL-3-dependent murine myeloid cell line 32D.cl3 called 32D.cl8.6 that does not express endogenous G-CSF-R. Surface expression of the G-CSF-R was determined using fluorescence-activated cell sorter analysis, and cell lines expressing equivalent levels of receptor were selected for further analysis, with at least three independent clones studied for each construct. Examples of clones expressing WT or mutant G-CSF-R proteins are shown in Fig. 1*B*.

Receptor Tyrosines Are Required for Proliferation and Differentiation but Are Dispensable for Survival at High Ligand Concentration—To ascertain whether receptor tyrosines were required at all to mediate proliferation, survival, and differentiation responses in response to G-CSF in 32D cells, we first compared the WT and tyrosine null mutant (mO) G-CSF-Rs. To this end, 32D[WT] and 32D[mO] transfectants were switched from IL-3- to G-CSF-containing medium following extensive washing to remove any traces of IL-3. In the absence of IL-3 or G-CSF, all transfectants died within 1 to 2 days without showing any signs of differentiation. However, in response to 100 ng/ml G-CSF, 32D[WT] cells showed transient proliferation for 5 to 7 days (Fig. 2). After 6 to 10 days, 32D[WT] cells developed into terminally differentiated neutrophils, as shown by an increased cytoplasm-to-nucleus ratio, a neutrophilic granule-containing cytoplasm, and lobulated nuclei (Fig. 3, *A* and *B*). In contrast, 32D[mO] cells showed only minimal proliferation and differentiation in response to G-CSF, although cell viability was largely maintained at this saturating ligand concentration (Figs. 2 and 3). This result establishes that receptor tyrosines are required to facilitate proliferation and differentiation responses from the full-length G-CSF-R.

Multiple Tyrosines Contribute to Proliferation and Differentiation Signaling—The almost complete abrogation of proliferation and differentiation signaling from the tyrosine null mutant receptor provided the opportunity to determine the contribution of individual tyrosines to these pathways through the analysis of clones expressing the tyrosine add back mutants (Figs. 2 and 3). This revealed that Tyr-704 alone (mA) could contribute to both G-CSF-mediated proliferation and differentiation signaling. This mutant elicited initial proliferation responses equivalent to those from the wild-type receptor, although the proliferative phase terminated slightly earlier. Concurrently, mA produced an earlier and more complete differentiation compared with the WT receptor. In contrast, Tyr-729 (mB) gave only a very weak signal for differentiation, with no significant increase in proliferation compared with the null mutant. The Tyr-744-containing mutant (mC) produced a phenotype similar to the Tyr-704-containing mA, although with slightly less proliferation and differentiation. Finally, analysis of 32D[mD] cells revealed that Tyr-764 projects strong proliferative signals, such that these cells continued to proliferate indefinitely on G-CSF, with no significant differentiation observed.

The Membrane-distal Region of the Human G-CSF-R Contains a Tyrosine-independent Differentiation Domain Essential for Neutrophilic Differentiation in 32D Cells—We had previously identified the membrane-distal domain of the G-CSF-R as essential for the transduction of differentiation signals based on the observation that a truncation mutant isolated from a patient with severe congenital neutropenia, mDA, which lacks this domain, was unable to elicit differentiation in myeloid cells (19, 25–27). The construction of mutant mA, which lacks all three tyrosines in this domain, allowed an assessment

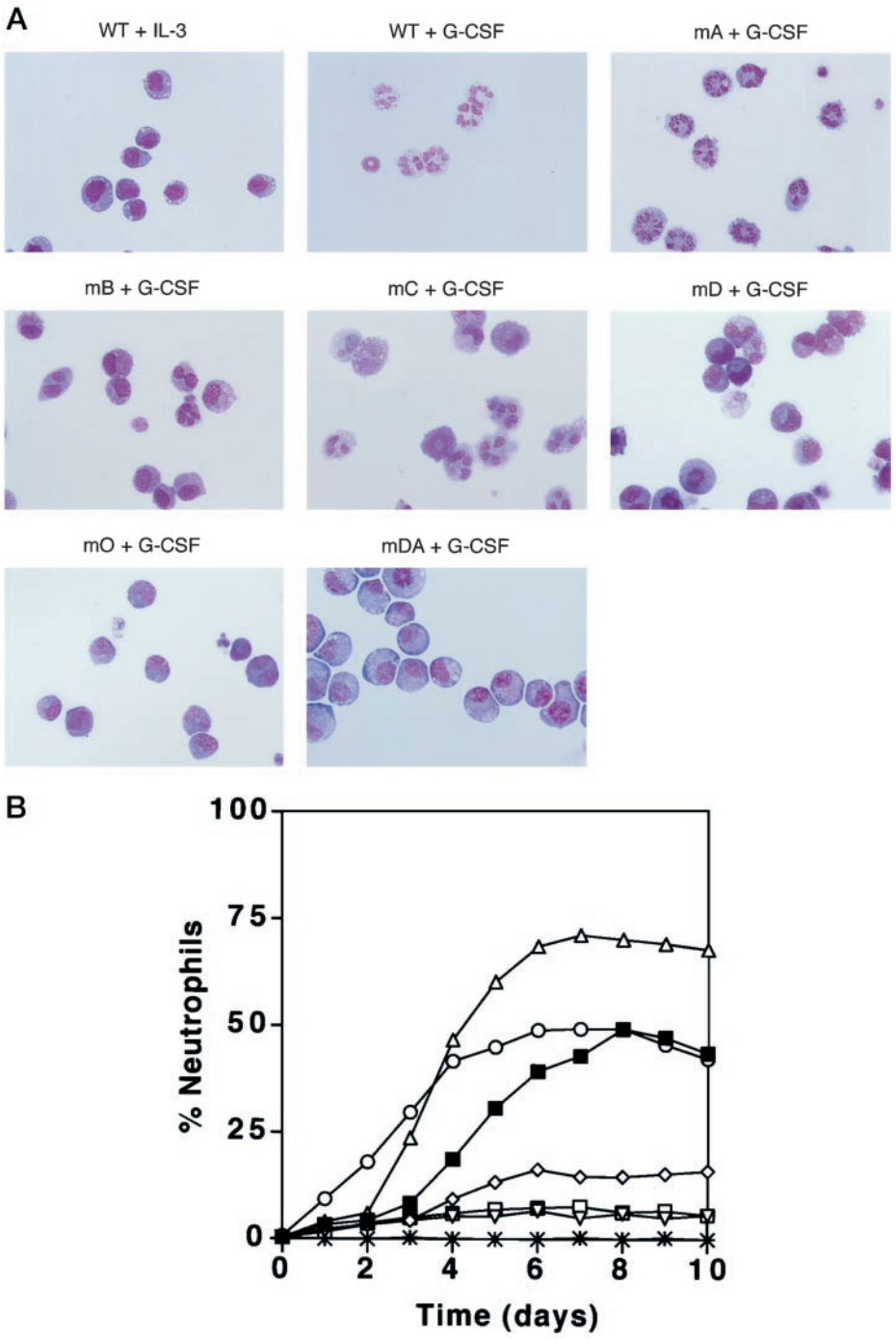

FIG. 3. **Neutrophilic differentiation of 32D.cl8.6 transfectants in response to G-CSF.** *A*, morphological features of 32D[WT] cells in the presence of IL-3 or representative clones expressing wild-type or mutant G-CSF-R after 7 days of exposure to G-CSF. *B*, maturation of 32D.cl8.6 cells expressing wild-type or mutant G-CSF-Rs, as indicated, expressed as the percentage of living cells showing terminal differentiation (% neutrophils) at each time point. Data represent the mean of three independent clones of each. ■, WT; △, mA; ◇, mB; ○, mC; ▽, mD; □, mO; ✶, mDA.

of whether tyrosines were required for these effects. Comparison of the 32D[mA] and 32D[mDA] clones revealed that the membrane-distal domain lacking all tyrosines still augments differentiation (Figs. 2 and 3).

Signals for Survival at Low Ligand Concentration Parallel Those for Differentiation—We have shown above that 32D[mO] cells are largely able to survive at 100 ng/ml G-CSF, without undergoing significant proliferation. In contrast, survival at lower G-CSF concentrations was impaired relative to 32D[WT] cells, such that 32D[WT] cells can survive on 0.1 ng/ml G-CSF, whereas 32D[mO] cells cannot (Fig. 4). We could then determine the contribution of individual tyrosines to this low dose G-CSF-mediated survival signaling by exposing clones to different concentrations of ligand. Results with the add-back mutants showed a strong role for Tyr-704 and Tyr-744 and a weak role for Tyr-729 in mediating survival, with Tyr-764 apparently having no role. This clearly parallels the effects of these tyrosines in mediating differentiation signals.

STAT3 Activation Correlates with Both Survival at Low Ligand Concentration and Differentiation—The concordant pattern of survival at low doses of G-CSF and differentiation resembled that for STAT3 activation from the G-CSF-R, which we have recently reported in Ba/F3 cells (18). In the various 32D cells clones, G-CSF-mediated STAT3 activation, as measured by both tyrosine and serine phosphorylation, was greatest with mA (Tyr-704) and mC (Tyr-744), with a lesser contribution from mB (Tyr-729) (Fig. 5). In contrast, mO was only weakly able to activate STAT3, whereas mD (Tyr-764) showed no more activation than mO. Thus, the ability of mutants to activate STAT3 showed a striking correlation with the strength of their respective differentiation and survival signals, suggesting a positive role for STAT3 in the control of both processes.

Identification of Pathways Important for Proliferative Responses from the G-CSF-R—Finally, we also sought to identify which signaling molecules might play a role in the tyrosine-mediated proliferation signaling from the G-CSF-R, *i.e.* via Tyr-704, Tyr-744, and Tyr-764. To achieve this we employed a novel strategy to identify SH2 domains that could interact directly with the phosphorylated tyrosines of the G-CSF-R. As an initial screen, we used a ^{32}P-labeled, tyrosine-phosphorylated WT G-CSF-R cytoplasmic domain simulating an activated receptor to probe a range of SH2 domains immobilized on nitrocellulose (Fig. 6*A*). This showed that the isolated SH2 domains of SHP-2 and Shc could bind to the receptor. In addition, full-length Grb2 could bind to the receptor, whereas a specific SH2 domain mutant of Grb2 could not, implying that the Grb2 SH2 domain also interacts directly with the G-CSF-R. We were next able to map the specific tyrosine binding site(s) of these SH2 domains by probing them with tyrosine-phosphorylated receptor proteins generated from the single tyrosine add-back mutants (Fig. 6*B*). This revealed direct binding of SHP-2, preferentially via its N-terminal SH2 domain, to both Tyr-704 and Tyr-764, and Grb2 and Shc via their respective SH2 domains to Tyr-764. Because these tyrosines also elicit strong proliferation signals, we can posit a direct role for SHP-2, Grb2, and Shc in mediating these responses. No interactions of these molecules with Tyr-744 were identified.

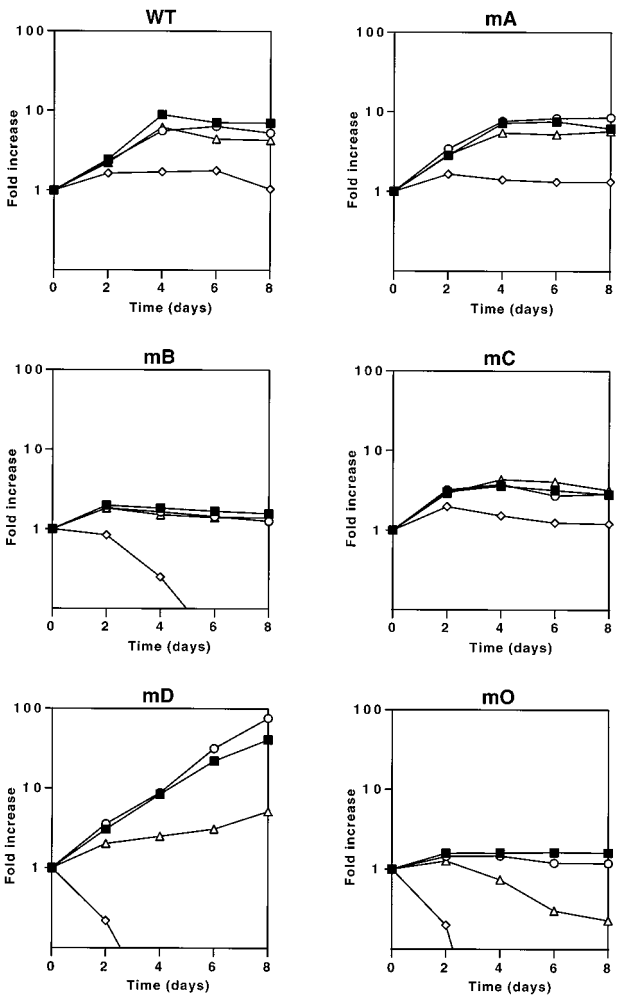

FIG. 4. **Survival signaling from G-CSF-R tyrosine mutants.** Cell proliferation/survival analysis of 32D clones expressing the indicated G-CSF-Rs performed as described in Fig. 2, except at 100 (■), 10 (○), 1 (△), and 0.1 (◇) ng/ml G-CSF.

DISCUSSION

Previous studies to determine the role(s) of the cytoplasmic tyrosines of the G-CSF-R in eliciting cellular responses have been performed with deletion and single tyrosine substitution mutants. These studies have yielded ambiguous results. This is likely due, at least in part, to the use of inappropriate cell models, which are unable to recapitulate the complete gamut of G-CSF responses (15, 17, 21, 22, 29, 34). Furthermore, use of truncation mutants to make inferences about tyrosine-specific effects (17, 21, 34) is also problematic because of altered receptor trafficking of such mutants (19, 39). Analysis of single Tyr → Phe substitution mutants has provided some insight into the function of the receptor tyrosines, although even here the results are somewhat conflicting, implicating Tyr-44 and, to a lesser extent, Tyr-704 and Tyr-729 in the macrophage differentiation of M1 cells (15), Tyr-703 or Tyr-728 of the murine G-CSF-R (equivalent to Tyr-704 and Tyr-729 of the human receptor) in the neutrophilic differentiation of L-GM-1 cells (29), and Tyr-764 in the proliferative responses of 32D cells (33). However, a major problem with these studies is that they may fail to reveal redundant pathways emanating from these tyrosines.

Analysis of tyrosine null and add-back mutants has provided a useful approach to investigate the complex roles of individual cytoplasmic tyrosine residues in cytokine receptor signaling (40, 41). Therefore, we used this approach to delineate the function of tyrosine-mediated pathways from the G-CSF-R. In addition, we chose a cell system, myeloid 32D cells, which most closely mimics *in vivo* differentiation, because these cells are able to differentiate from blast-like cells into mature neutrophils. The results of this analysis revealed multiple signals emanating from the receptor tyrosines to control proliferation, differentiation, and survival (Fig. 7). In addition, these studies show that signals independent of receptor tyrosines also contribute to differentiation and survival.

We found that signals from the G-CSF-R for both survival at low G-CSF concentration and differentiation largely overlapped, being mediated most strongly by Tyr-704 and Tyr-744, with a lesser contribution from Tyr-729. This showed a close correlation with the ability of these tyrosines to activate STAT3 in these cells. The involvement of STAT3 in G-CSF-mediated granulocytic differentiation is consistent with a recent study showing that expression of dominant-negative STAT3 could totally block this process (42). However, our data also suggest that STAT3 activation may represent the key pathway in maturation signaling from the full-length G-CSF-R. Furthermore, we provide evidence suggestive of a role for STAT3 in G-CSF-dependent survival signaling, as has also been postulated for the related gp130 receptor component (43). However, it is clear

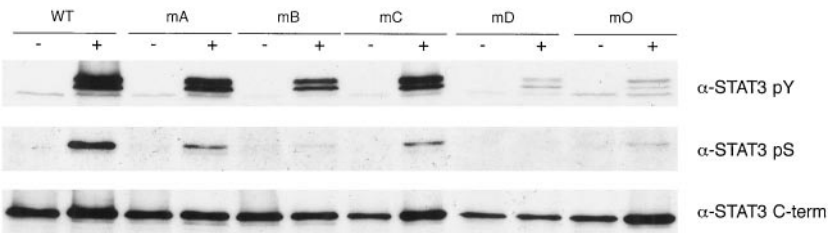

FIG. 5. **STAT3 activation by G-CSF-R tyrosine mutants.** Western blot analysis of total lysates from 32D cells expressing wild-type or mutant G-CSF-Rs, either starved (−) or stimulated with G-CSF for 10 min (+), probed with the antibodies indicated. *pY*, Tyr(P); *pS*, Ser(P); *C-term*, C terminus.

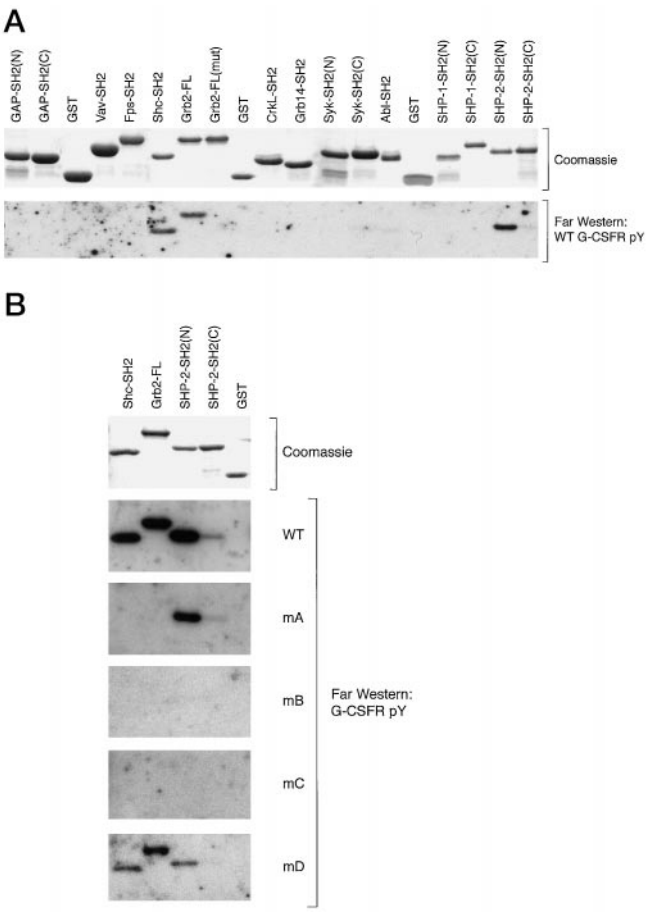

FIG. 6. **Far Western analysis of SH2-domains with recombinant tyrosine-phosphorylated G-CSF-R.** *A*, glutathione *S*-transferase (*GST*) fusions of various SH2 domain-containing signaling molecules were separated on replicate SDS-polyacrylamide gel electrophoresis gels and either stained with Coomassie or transferred to nitrocellulose for Far Western analysis with ^{32}P-labeled, tyrosine-phosphorylated (*Tyr(P)*) WT G-CSF-R cytoplasmic domain as a probe. *B*, far Western as described in *A*, except using as probes ^{32}P-labeled, tyrosine-phosphorylated cytoplasmic domains from WT or mutant G-CSF-Rs, as indicated.

that, at high G-CSF concentrations, tyrosine-independent survival mechanisms also operate, potentially involving phosphatidylinositol 3-kinase (34) or mitogen-activated protein kinase (21). Finally, there appears to be subtle differences in G-CSF-mediated STAT3 activation in 32D cells compared with that we have reported in Ba/F3 cells (18), such that the contribution of tyrosine-independent STAT3 activation is less important, whereas the Tyr-729-mediated route represents a novel pathway of STAT3 activation in 32D cells. The reason for these cell-specific differences remains unclear.

Proliferation signaling from the G-CSF-R was predominantly mediated via Tyr-704 and Tyr-764, with a minor contribution from Tyr-744. We have previously reported formation of Grb2/SHP-2, Grb2/p90, and Grb2/Shc/p140 complexes in response to G-CSF, the latter two being dependent on Tyr-764 (22). Using *in vitro* binding studies, we show that Tyr-704 is a direct docking site for SHP-2, whereas Tyr-764 is a direct docking site for SHP-2, Grb2, and Shc, largely consistent with the published consensus binding sites for these molecules (44, 45). In addition, we and others have shown that Grb2 interacts with SHP-2 and Shc via binding of its SH2 domain with phosphorylated tyrosines in the SHP-2 C terminus (24, 46) and at position 317 of Shc (31), respectively. Thus, there appears to be at least four mechanisms by which Grb2 can interact with the activated G-CSF-R: directly via Tyr-764 (complexed with p90?) or indirectly via SHP-2 at Tyr-704 and Tyr-764 or Shc at Tyr-764. The relative role of these alternate complexes in mediating G-CSF responses remains an important consideration for future investigations. However, because SHP-2, Grb2, and Shc have all been implicated in proliferation (31, 47–50), it seems likely that these are the molecules responsible for the proliferative signals emanating from Tyr-704 and Tyr-764. The molecule(s) docking to Tyr-744, which mediate its weak proliferation signal remain to be elucidated.

We have previously identified the membrane-distal domain of the G-CSF-R as being essential for the transduction of differentiation signals (19, 25–27). Analysis of the 32D[mA] clones in this study has revealed that the membrane-distal domain is required for differentiation independent of receptor tyrosines (Figs. 2 and 3). However, because the 32D[mO] clones differentiated poorly, we can presume that this domain alone is

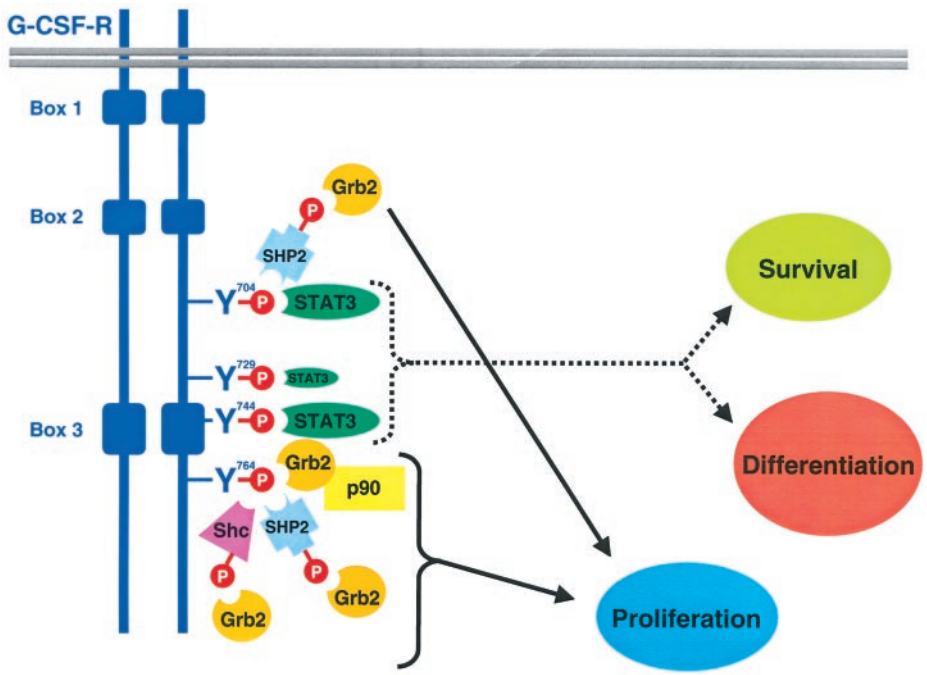

FIG. 7. **Model for signaling from the G-CSF-R leading to specific cellular responses.** Potential tyrosine-dependent pathways leading to proliferation, differentiation, and survival are indicated. In addition, it is obvious that tyrosine-independent pathways also contribute to these processes, although the mechanism(s) remain unclear.

insufficient to induce complete maturation but rather co-operates with additional tyrosine-dependent signals (such as from Tyr-704 or Tyr-744). We have recently shown that this region is important for receptor internalization and concomitant deactivation (19). Therefore, it is likely that a major function of this tyrosine-independent differentiation domain is to negatively regulate proliferation. Consistent with this, mD, which contains this domain, elicits sustained proliferation but at a reduced rate compared with mDA, which does not. In addition, the minor activation of STAT3 by this domain could also contribute to its differentiation-inducing function.

The data presented here has unequivocally assigned specific roles to the tyrosines of the G-CSF-R in mediating differentiation, proliferation, and survival in a myeloid system capable of full neutrophilic differentiation. We have shown that the four tyrosines of the G-CSF-R possess distinct, yet overlapping, functions. This is a similar conclusion to that obtained from recent studies with other cytokine receptors, such as the erythropoietin receptor (41) and the common β-chain of the granulocyte-macrophage colony-stimulating factor receptor (40). Having now identified the key intracellular mediators downstream of the receptor tyrosines, future studies will aim to identify the targets of these molecules in eliciting the various G-CSF-mediated biological responses.

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