

Differential influence of tyrosine residues of the common receptor β subunit on multiple signals induced by human GM-CSF

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Background: GM-CSF induces the proliferation and differentiation of a broad range of hematopoietic cells. Stimulation with this cytokine results in the induction of rapid tyrosine phosphorylation of cellular proteins, including the common β subunit of its receptor. However, the significance of β subunit phosphorylation with regard to regulation of signaling molecules and the functions of GM-CSF is less well understood. **Objective:** To investigate the role of cytoplasmic tyrosine residues, we performed mutational analyses of the β subunit. **Methods:** A series of β subunit mutants with 1, some, or all of 8 cytoplasmic tyrosines converted to phenylalanines were constructed. These β subunit mutants were expressed in murine BA/E3 cells together with the wild-type human α subunit, and their potentials to transmit signals were examined. **Results:** A mutant β subunit lacking all cytoplasmic tyrosines (Fall) activated JAK2, but not Shc, SHP-2, the Raf/ERK cascade, signal transducer and activator of transcription 5 (STAT5), or the *c-fos* promoter, nor did it effectively induce proliferation. Adding back each tyrosine to Fall revealed that (1) Tyr577 was necessary and sufficient for Shc phosphorylation; (2) Tyr577, Tyr612, and Tyr695 were involved in activation of SHP-2, the Raf/ERK cascade, and *c-fos* transcription; and (3) all tyrosines, but particularly Tyr612, Tyr695, Tyr750, and Tyr806, facilitated STAT5 activation. Impaired cell growth was also partly restored by any of the tyrosines. Interestingly, the Fall mutant was still capable of suppressing apoptosis and maintaining cell viability.

Conclusion: These results provide evidence that β subunit tyrosines possess distinct, yet overlapping, roles in the activation of multiple signaling pathways induced by GM-CSF. In addition, GM-CSF may activate both β subunit tyrosine-dependent and -independent pathways, with the latter being sufficient to transmit survival signals. (*J Allergy Clin Immunol* 1999;103:S462-70.)

Key words: Signal transduction, common β subunit, tyrosine phosphorylation, SH2 and PTB domains

GM-CSF is a pleiotropic cytokine that supports the proliferation, survival, and differentiation of hematopoietic progenitor cells and the enhancing multiple functions of

Abbreviations used

β c:	Common β subunit
hGM-CSF:	Human GM-CSF
mIL-3:	Murine IL-3
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
SH2:	Src homology 2
SRE:	Serum response element
STAT:	Signal transducer and activator of transcription

mature neutrophils, macrophages, and eosinophils.^{1,2} The functional high affinity receptor for GM-CSF is composed of α and β subunits, both belonging to the type I cytokine receptor superfamily.³⁻⁶ The β subunit, which is also shared by the IL-3 and IL-5 receptors (thereby being termed the *common β subunit* [β c]), has a relatively large cytoplasmic domain and plays a pivotal role in signal transduction.⁷

GM-CSF induces a proliferative response and the expression of early-response genes such as *c-fos*, *c-jun*, and *c-myc* in target cells.⁷ Stimulation with GM-CSF results in rapid and reversible tyrosine phosphorylation of multiple proteins, including the GM-CSF receptor proteins.^{8,9} The importance of tyrosine phosphorylation in GM-CSF signaling has been suggested by the results of various experiments with tyrosine kinase inhibitors.⁹⁻¹¹ Neither the α nor the β c subunits of the receptor possesses an intrinsic tyrosine kinase domain. Instead, several tyrosine kinases, including JAK2,¹² have been shown to be associated with the receptor complex and are activated by GM-CSF stimulation. A series of experiments with dominant negative JAK2 mutant revealed that JAK2 activity is necessary for all the biologic functions of GM-CSF.¹³ For JAK2 activation, the membrane-proximal region of β c containing the box 1 motif is necessary and sufficient.^{12,14} On the other hand, for induction of the *c-fos* gene, not only JAK2 but also a membrane-distal region of β c containing several tyrosine residues is also required.¹⁵ Because GM-CSF stimulation results in tyrosine phosphorylation of β c^{8,9} and proteins with Src homology 2 (SH2) and/or phosphotyrosine-binding domain(s), such as Shc,¹⁶ SHP-2,¹⁷ Vav,¹⁸ c-Cbl,¹⁹ and STAT5,²⁰ the β c tyrosines may have a role in the signaling process. We previously demonstrated¹⁵ that GM-CSF-induced activation of the *c-fos* promoter by β 589, a truncated β c mutant, was significantly diminished by the substitution of a single tyrosine at position 577 (Tyr577),

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thereby indicating an important role of this tyrosine in signaling. However, the full-length β c with the same mutation at Tyr577 transduced sufficient signals to activate the *c-fos* promoter, suggesting that other functional domains, probably also tyrosine residues, play a similar role. We also found that β c undergoes phosphorylation of multiple tyrosines, including Tyr577. These observations strongly imply the involvement of other β c tyrosine residues in the transmission of GM-CSF signals.

To better understand the pathways and regulatory mechanisms of GM-CSF signaling, we examined the role of cytoplasmic tyrosines of the human β c, by using various mutants with 1 or more of these residues replaced by phenylalanine. The signaling potential of these mutants was analyzed after reconstitution of high-affinity receptors by combination with the wild-type α subunit in murine BA/F3 cells. Our evidence shows that all 8 β c tyrosines are important in transmitting GM-CSF signals for cell proliferation, with each one possessing distinct yet overlapping functions.

METHODS

Cells and culture

A murine IL-3 (mIL-3)-dependent pro-B cell line, BA/F3, was maintained in RPMI-1640 medium supplemented with 5% FCS, 50 U/mL penicillin, 50 μ g/mL streptomycin, and 0.25 ng/mL mIL-3. Factor depletion was performed with the same medium without mIL-3 in cultures maintained at 37°C for 5 to 6 hours. Endogenous murine β c (AIC2B) in BA/F3 cells can interact functionally with the human GM-CSF (hGM-CSF) receptor α subunit at high hGM-CSF concentrations (≥ 100 ng/mL) and can support proliferation.⁶ In this study, we used 10 ng/mL of hGM-CSF for stimulation unless otherwise indicated. Purified recombinant hGM-CSF produced in *Escherichia coli* was provided by Schering-Plough K.K. (Osaka, Japan). mIL-3 expressed in silkworm (*Bombyx mori*) was purified as described elsewhere.²¹

Antibodies

Rat mAb against human common β c was prepared as described elsewhere²² and used for FACS analysis. An antiphosphotyrosine mAb (clone 4G10, 05-321) and antiserum against Shc (06-203) were obtained from Upstate Biotechnology, Inc (Lake Placid, NY); antiserum against SHP-2 (sc-280) was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif), and an mAb against GRB2 (clone 3F2, MS-20-3) was from Medical and Biological Laboratory Co Ltd (Nagoya, Japan). A rabbit polyclonal antibody against STAT5 (N1; recognizing both STAT5A and STAT5B) was provided by Dr Hiroshi Wakao (Helix Research Institute, Kisarazu, Japan).

Plasmids

A *c-fos* promoter-luciferase gene fusion plasmid containing the human *c-fos* promoter fragment (−404 to +41) fused to the luciferase fragment was constructed as described elsewhere.²³ A β -casein promoter-luciferase construct, consisting of a luciferase gene under the control of a minimal β -casein promoter,²⁰ was provided by Dr Hiroshi Wakao (Helix Research Institute, Kisarazu, Japan).

hGM-CSF receptor β c (KH97) cDNA was originally cloned into the pME18S vector, as described previously.⁵ The β c mutants used in this study were constructed by introducing point mutations with sequential PCR procedures²⁴ with appropriately designed oligonucleotide primers. The codons for tyrosine, TAC (at 450, 452, 577, 612 and 866) and TAT (at 695, 750 and 806), were changed to

codons for phenylalanine (TTC and TTT, respectively). The PCR-amplified fragments, alone or in combination, were then used to replace the tyrosine-containing sequences of wild-type β c in the pME18S vector, with the use of intrinsic restriction enzyme sites within the cDNA. The accuracy of all nucleotide sequences of the fragments derived from PCR reaction was confirmed by dideoxy sequencing with an automated sequencer (Applied Biosystems, Warrington, UK).

Establishment of stable transfectants

With electroporation, the β c expression plasmid (13.5 μ g) was transfected together with the pME18S vector containing a neomycin resistance gene (1.5 μ g) into BA/F3 cells, which stably expressed the wild-type hGM-CSF receptor α subunit, as described previously.¹⁵ After selection with G418 (1 mg/mL) for about 10 days, surface expression of the transfected β c gene product by G418-resistant clones was confirmed by flow cytometry with an FACSscan (Becton Dickinson, San Jose, CA) and an anti- β c antibody. At least 2 independent clones were established for each of the β c mutants and were used in this study.

Transient transfection and reporter gene assays

Plasmid DNAs were transiently transfected into BA/F3 cells by electroporation. Each transfection used 3 μ g of various β c mutant cDNAs or control vector (pME18S), in combination with either *c-fos*-luciferase (3 μ g) or β -casein-luciferase (15 μ g). BA/F3 cells, which stably express the wild-type hGM-CSF receptor α subunit (3×10^6 cells), were washed twice with OPTI-MEM (Life Technologies, Inc, Grand Island, NY), resuspended in 200 μ L of OPTI-MEM, and then mixed with plasmid DNAs. An electronic pulse was then applied with a Gene Pulser (Bio-Rad, Hercules, Calif) set at 960 μ F and 200 V. Cells were recovered and maintained in the presence of mIL-3 for about 12 hours and then separated into 3 aliquots. After factor depletion for 6 hours in mIL-3-free medium containing 5% FCS, the cells were stimulated with hGM-CSF (10 ng/mL), mIL-3 (1 ng/mL), or no cytokine for the specified period and then harvested. Protein was extracted by 3 cycles of freezing and thawing, and the protein concentration was determined with the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. Luciferase activity was measured with a luminometer (model LB9501; Berthold Lumat Co Ltd) and the luciferase assay substrate (Promega, Madison, Wis).

Preparation of protein samples and Western blot analysis

To analyze tyrosine phosphorylation of JAK2, Shc, SHP-2, or STAT5, immunoprecipitation was performed with the corresponding antibody, as described previously.¹⁵ Protein samples were electrophoresed on SDS-polyacrylamide gel, electrophoretically transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, Mass), and then subjected to Western blot analysis, with appropriate antibodies and an enhanced chemiluminescence detection reagent (Amersham, Buckinghamshire, UK), as described previously.¹⁵

Cell-proliferation assays

Short-term cell proliferation was measured by the colorimetric assay of Mosmann²⁵ with the use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described previously.¹⁵

To analyze long-term proliferation, BA/F3 transfectants were deprived of mIL-3 for 6 hours and then cultured in the absence or presence of either hGM-CSF (10 ng/mL) or mIL-3 (1 ng/mL). Viable cells were counted by the trypan blue dye exclusion method.

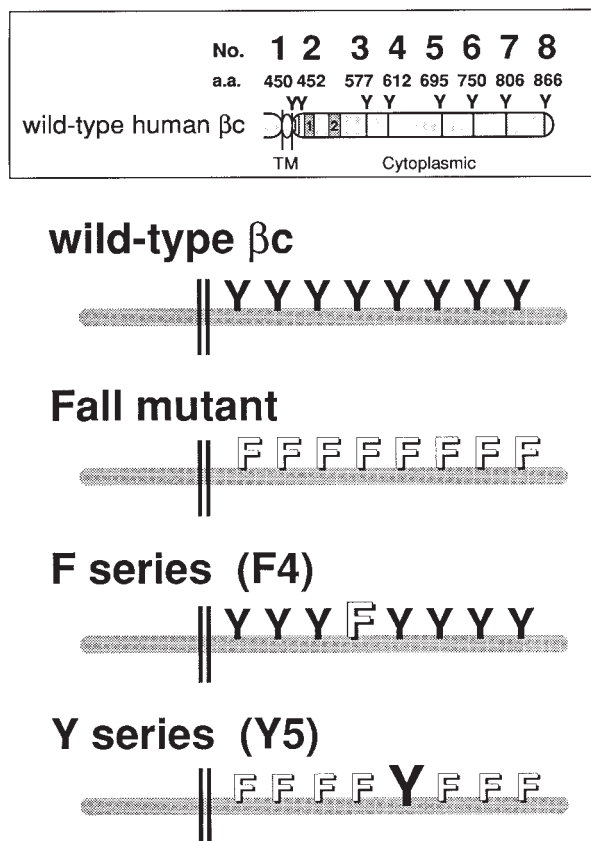


FIG 1. Diagram of the βc mutants used in this study. Y and F denote tyrosines and introduced phenylalanines, respectively. The structure of wild-type βc (with the extracellular portion abbreviated) is shown at top (boxed). TM, transmembrane region; 1 and 2, the box 1 and box 2 motifs, respectively. Note that the F3 mutant was previously termed β wild;Y577F.^{13,15}

Statistical analysis

The results were expressed as mean $\pm$ SD. Statistical significance was determined by Scheffé's multiple-comparison test, with $P < .05$ considered to be statistically significant.

RESULTS

We previously demonstrated that Tyr577 of human βc plays an important role in GM-CSF receptor signaling.¹⁵ To investigate the possible involvement of other βc tyrosines in GM-CSF signaling, we performed a mutational analysis of all 8 βc cytoplasmic tyrosines by phenylalanine substitution. We constructed 3 types of the βc mutants (Fig 1): the "Fall" mutant with substitution of all 8 tyrosines; "F series" mutants containing only a single (or 2, in the case of F12) mutant tyrosine with the remaining residues intact; and "Y series" mutants possessing only a single (or 2, in the case of Y12) intact tyrosine residue with mutation of the remaining tyrosines. To analyze the molecules involved in GM-CSF signaling, we established stable transfectants of BA/F3 cells expressing hGM-CSF receptors composed of the wild-type α subunit and βc mutants.

We first checked the activation of tyrosine kinase JAK2, which is a critical step in receptor activation.¹³ Immunoprecipitation and Western blotting showed that all βc mutants, including Fall, could activate JAK2, as judged from the tyrosine phosphorylation of this molecule (data not shown). Therefore βc tyrosine residues were not required for JAK2 activation.

Tyr577, Tyr612, and Tyr695 transmit signals leading to *c-fos* promoter activation

We next analyzed the role of βc tyrosines in activation of the *c-fos* promoter using a transient transfection assay. A reporter plasmid carrying the *c-fos* promoter fused to the luciferase gene was cotransfected with receptor cDNAs. After stimulation with hGM-CSF, cell lysates were prepared and luciferase activity was determined. Substitution of all 8 tyrosines (the Fall mutant) markedly diminished the transduction of *c-fos* activation signals by βc (Fig 2, A). In contrast, all the F series mutants, which had only 1 or 2 (F12) mutant tyrosine residues, activated the *c-fos* promoter (Fig 2, A). Thus hGM-CSF-induced activation of the *c-fos* promoter was mediated through multiple βc tyrosines. We then used the Y series mutants, each of which had only 1 or 2 (Y12) normal tyrosine residues, to assess whether each residue alone could transmit signals leading to activation of the *c-fos* promoter. As shown in Fig 2, B, the Y3 mutant with Tyr577 as the sole cytoplasmic tyrosine activated the *c-fos* promoter to a level comparable to that seen with wild-type βc , a finding consistent with our previous results for the truncation mutant β 589.¹⁵ In addition, the Y4 and Y5 mutants (possessing Tyr612 and Tyr695, respectively) also partly activated the promoter. Therefore 3 distinct βc tyrosine residues, namely Tyr577, Tyr612, and Tyr695, independently transduced signals activating the *c-fos* promoter.

Because a dominant-negative Ras mutant can inhibit GM-CSF-induced activation of the *c-fos* promoter,²⁶ it seems likely that the signaling pathway from the receptor to the promoter involves Ras activation. We previously analyzed 2 signaling molecules, Shc and SHP-2, which are positive regulators of Ras activation.¹⁵ We and others^{15,27} have shown that Tyr577 is necessary for induction of Shc phosphorylation; as seen in Fig 3, A, the F3 mutant lacking Tyr577 failed to induce Shc phosphorylation and subsequent association with the adaptor molecule GRB2. We then investigated whether the Y3 mutant (with Tyr577 as the only cytoplasmic tyrosine) was able to mediate this event. As expected, this mutant induced tyrosine phosphorylation of Shc and its association with GRB2 (Fig 3, A), demonstrating that Tyr577 alone was sufficient for Shc phosphorylation. Because Tyr612 and Tyr695 can also independently transduce signals activating the *c-fos* promoter, this result further implies the involvement of Shc-independent pathway(s) in GM-CSF-induced Ras activation.

We previously showed that tyrosine phosphorylation of SHP-2 is mediated by Tyr577 and by other functional sites.¹⁵ To assess the possible involvement of SHP-2 with Tyr612 and/or Tyr695, which positively regulate *c-fos* promoter activation, tyrosine phosphorylation of SHP-2 in

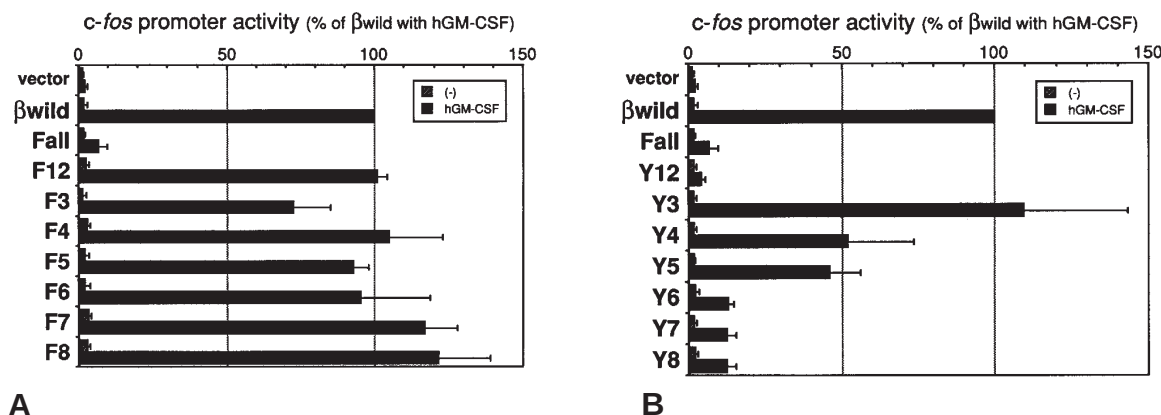


FIG 2. Tyr577, Tyr612, and Tyr695 transmit signals leading to activation of the *c-fos* promoter. Activation of *c-fos* promoter by mutant β c (A, F series mutants; B, Y series mutants) was measured by a transient transfection assay. The *c-fos* promoter-luciferase fusion construct was cotransfected with mutant β c cDNA into BA/F3 cells expressing the wild-type hGM-CSF receptor α subunit. After 6 hours of factor depletion, cells were left unstimulated or were stimulated with 10 ng/mL hGM-CSF or 1 ng/mL mIL-3 for 6 hours at 37°C. Cell lysates were prepared, and luciferase activity was measured with a luminometer. The *c-fos* promoter activity was calculated by dividing the luminescence (relative light units/min/ μ g of total protein) without stimulation or with hGM-CSF stimulation by that with mIL-3 stimulation, and results are presented as a percentage of the value with wild-type β c (β wild). Values are the average of at least 3 experiments, and SDs are shown as error bars. Statistical significances of the differences in the promoter activities between hGM-CSF-stimulated samples were as follows: wild-type β c and Fall or any of the Y series mutants except for Y3, significant at $P < .01$; Y3 and Y4 or Y5, significant at $P < .01$. (Adapted from Itoh, T., et al. Definition of the role of tyrosine residues of the common β subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor. *Mol Cell Biol* 1998;18:742-52. Copyright© 1998, The American Society for Microbiology. All rights reserved.)

BA/F3 transfectants was analyzed (Fig 3, B). Among the Y series mutants, Y4 induced tyrosine phosphorylation of SHP-2 at a level similar to that induced by wild-type β c. SHP-2 phosphorylation was also induced by Y3 and Y5, although at a slightly lower level. This phosphorylation was associated with coimmunoprecipitation of GRB2 and SHP-2, implying a relationship with Ras activation. These observations showed that Tyr612 and Tyr695, in addition to Tyr577, could induce tyrosine phosphorylation of SHP-2 and its subsequent association with GRB2.

To confirm that SHP-2 phosphorylation resulted in activation of the Ras pathway, we examined the activation of molecules known to function downstream of Ras. We analyzed the phosphorylation of serine/threonine kinases Raf-1 and ERK2 by mobility shift SDS-PAGE and found that the Y3, Y4, and Y5 mutants, which induced SHP-2 phosphorylation, also induced phosphorylation of Raf-1 and ERK2 (data not shown). One of the nuclear targets phosphorylated by activated ERKs is the ternary complex factors,²⁸ which bind to the serum response element (SRE) in the *c-fos* promoter region and activate transcription of the *c-fos* gene. Our recent study of the *c-fos* promoter region also revealed a vital role for the SRE in hGM-CSF-induced transcriptional activation of the *c-fos* gene.²⁶ In this study, we also performed transient transfection assays using the SRE-CAT construct as a reporter gene and confirmed that the Y3, Y4, and Y5 mutants were all capable of stimulating SRE site-driven transcriptional activation (data not shown).

β c tyrosine residues are required for STAT5 activation

Extensive studies about various cytokine receptor systems have established that STAT family transcription fac-

tors play a critical role in cytokine signaling.^{29,30} Among the STAT family members, GM-CSF activates STAT5 in BA/F3 cells.²⁰ In the case of other cytokines such as IL-2, erythropoietin, or growth hormone, activation of STAT5 has been shown to depend on specific tyrosine residues of their receptor subunits.³¹⁻³⁶ Thus we examined the possibility that β c tyrosines also play a role in the activation of STAT5 by GM-CSF. Tyrosine phosphorylation of STAT5, which is required for dimer formation, was assessed by immunoprecipitation and subsequent Western blotting. As shown in Fig 4, A, induction of STAT5 phosphorylation was significantly diminished when all 8 β c tyrosines were substituted (Fall mutant), thereby demonstrating the essential role of tyrosine residues in β c-mediated phosphorylation of STAT5. To further define the tyrosines involved, we analyzed Y series mutants and found that Y4, Y5, Y6, and Y7 were capable of inducing STAT5 phosphorylation to nearly the same extent as the wild-type β c. Moreover, the level of phosphorylation induced by Y12, Y3, and Y8 was significantly higher than that induced by Fall. These data suggested that any of the 8 tyrosines could independently contribute to activation of STAT5, albeit to a varying extent.

We further examined whether the observed tyrosine phosphorylation of STAT5 was correlated with its transactivation, by performing transient transfection assays using a reporter gene carrying the β -casein promoter that is responsive to STAT5. The transactivation capacity of STAT5 was found to depend on β c tyrosines in a manner similar, though not identical, to that seen with STAT5 phosphorylation (Fig 4, B). The Fall mutant achieved little expression of the reporter gene, although all of the Y series mutants (except Y12) were capable of inducing significantly higher expression. There was no marked

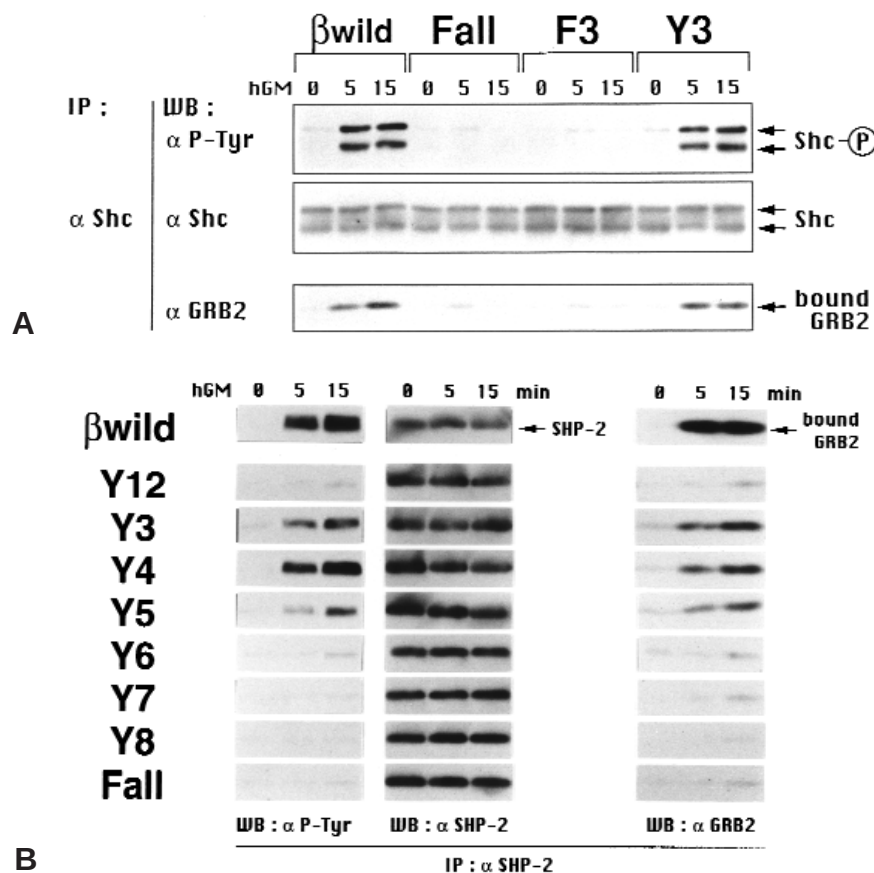


FIG 3. Requirement of β c tyrosine residues for phosphorylation and association with GRB2 of Shc (A) and SHP-2 (B). Factor-deprived BA/F3 stable transfectants (1×10^7 cells) were stimulated with 10 ng/mL hGM-CSF at 37°C for the indicated period. Cells were lysed, and immune complexes formed with anti-Shc or anti-SHP-2 antibodies were precipitated. Protein samples were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and subjected to Western blotting with antibodies against phosphotyrosine (4G10; top panel and left column in A and B, respectively), Shc (center panel in A), SHP-2 (center column in B), or GRB2 (bottom panel and right column in A and B, respectively). (B from Itoh, T., et al. Definition of the role of tyrosine residues of the common β subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor. *Mol Cell Biol* 1998;18:742-52. Copyright© 1998, The American Society for Microbiology. All rights reserved.)

difference between the Fall and Y12 mutants. These results indicated that GM-CSF-induced activation of STAT5 was largely dependent on multiple β c tyrosine residues. As expected, all F series mutants induced transactivation of the β -casein promoter to a level not significantly different from that seen with wild-type β c (Fig 4, C), indicating that none of the β c tyrosine residues was indispensable for this signaling event.

β c tyrosine residues are required for the maximal proliferative response to hGM-CSF, but not for maintaining cell viability

To investigate the roles of these signaling pathways in regulating the biologic functions of GM-CSF, we determined whether β c tyrosines were involved in transmitting growth-promoting signals. As shown in Fig 5, A, the potential of β c to induce short-term proliferation of BA/F3 cells (as detected by the MTT assay) was severe-

ly impaired in the absence of all cytoplasmic tyrosines (Fall mutant). This indicated that the β c tyrosines are necessary for GM-CSF-stimulated proliferation, at least to induce the maximal response. Further analysis with BA/F3 cells expressing Y series mutants showed that deficient proliferation was at least partly restored by the presence of any 1 (or 2, in the case of Y12) of the β c tyrosines (Fig 5, B). Particularly, the Y3, Y4, and Y5 mutants induced a stronger growth response than the other mutants (Fig 5, B). Therefore each of the β c tyrosines was able to independently and differently mediate growth signals.

To determine whether or not the lack of growth promotion by the Fall mutant was due to a defect in maintaining cell viability, we examined the long-term proliferation and survival of BA/F3 transfectants using trypan blue dye exclusion. In the presence of 10 ng/mL hGM-CSF, BA/F3 cells expressing the Fall mutant kept on proliferating at a lower growth rate and showed no signifi-

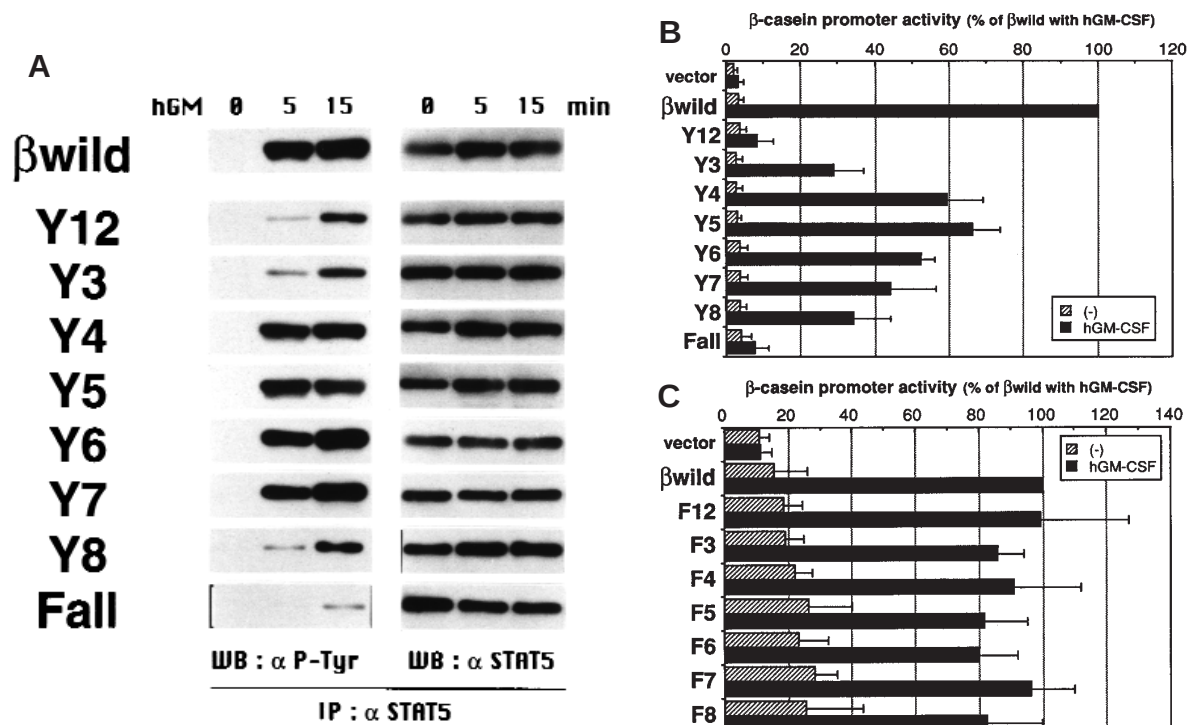


FIG 4. Tyrosine residues of βc are required for STAT5 activation. **A**, Tyrosine phosphorylation of STAT5. Factor-deprived BA/F3 stable transfectants (5×10^6 cells) were stimulated with 10 ng/mL hGM-CSF at 37°C for the indicated period. Cells were lysed, and immune complexes formed with anti-STAT5 antibodies were precipitated. Protein samples were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and subjected to Western blotting with antibodies against phosphotyrosine (4G10; left column) or STAT5 (right column). Activation of β -casein promoter by each mutant βc (**B**, Y series mutants; **C**, F series mutants) was measured by a transient transfection assay. The β -casein promoter-luciferase fusion construct was cotransfected with mutant βc cDNA into BA/F3 cells that expressed the wild-type hGM-CSF receptor α subunit. After 6 hours of factor depletion, cells were left unstimulated or were stimulated with 10 ng/mL hGM-CSF or 1 ng/mL mL-3 for 6 hours at 37°C. Cell lysates were prepared, and luciferase activity was measured with a luminometer. β -casein promoter activity was calculated by dividing the luminescence (relative light units/minute/micrograms of total protein) without stimulation or with hGM-CSF stimulation by that with mL-3 stimulation, and results are presented as a percentage of the value with wild-type βc ($\beta wild$). Values are the average of 3 experiments, and standard deviations are shown as error bars. Statistical significance of the differences in the promoter activities between hGM-CSF-stimulated samples were as follows: wild-type βc and Fall or any of the Y series mutants, significant at $P < .05$; Fall and Y4, Y5, Y6, or Y7, significant at $P < .05$; Y12 and Y8, not significant; wild-type βc and any of the F series mutants, not significant. (A, B from Itoh, T., et al. Definition of the role of tyrosine residues of the common β subunit regulating multiple signaling pathways of granulocyte-macrophage colony-stimulating factor receptor. *Mol Cell Biol* 1998;18:742-52. Copyright© 1998, The American Society for Microbiology. All rights reserved.)

cant loss of viability (Fig 6). The viability of the cells was maintained at the same level for more than 1 week (data not shown). Therefore the βc tyrosines are not necessary for transmitting the survival signal. Consistent with this finding, all the Y series mutants also supported cell viability (data not shown).

DISCUSSION

In this study, we focused on βc cytoplasmic tyrosine residues and analyzed their role in GM-CSF signaling. Substituting all 8 cytoplasmic tyrosines (Fall mutant) severely impaired the ability of βc to transduce various signals, thereby indicating the critical role of these tyrosines in signaling. Neither of the F series mutants, lacking only 1 tyrosine, showed any significant decrease in activation of the *c-fos* promoter and STAT5-mediated transcription or any significant loss of growth promotion

(Figs 2, A, and 4, C; data not shown), indicating that some (or all) of the βc tyrosines have overlapping functions. Therefore we constructed Y series mutants that added back each of the tyrosines lacking in the Fall mutant.

Among the 8 βc tyrosines, 3 (Tyr577, Tyr612 and Tyr695) were involved in and could individually induce SHP-2 phosphorylation, association with GRB2, and activation of the putative downstream Raf/ERK pathway resulting in transactivation of the *c-fos* gene through SRE, as depicted in Fig 7. Tyr577 was also necessary and sufficient for tyrosine phosphorylation of Shc, another molecule implicated in the activation of Ras through the GRB2/Sos complex. Thus Tyr577 uses both Shc and SHP-2 to stimulate this pathway, although Tyr612 or Tyr695 only induces phosphorylation of SHP-2. This may partly account for the finding that the Y3 mutant activated the *c-fos* promoter more strongly than the Y4 and Y5 mutants (Fig 2, B). Although we did not clearly observe coim-

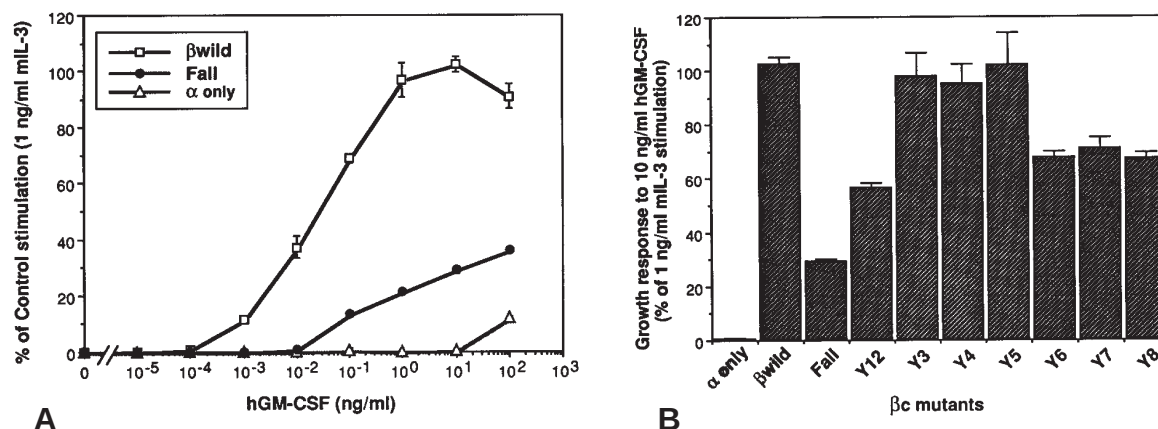


FIG 5. Tyrosine residues of β c are necessary for the maximal proliferative response to hGM-CSF. Short-term proliferation of BA/F3 transfectants that expressed the hGM-CSF receptor α subunit without β c (α only; **A**, **B**), with wild-type β c (β wild; **A**, **B**), with the Fall mutant (**A**, **B**), or with Y series mutants (**B**) are shown. The BA/F3 transfectants were subjected to factor deprivation for 6 hours and then cultured for 24 hours in the presence of 0 to 100 ng/mL hGM-CSF or 0 to 10 ng/mL mL-3. Cell growth was examined by the MTT assay. The vertical axis indicates the relative MTT reduction value normalized to the value for cells incubated with 1 ng/mL mL-3. In **B**, only the values obtained for stimulation with 10 ng/mL hGM-CSF are shown. All values are the average of 3 samples, and standard deviations are shown as error bars. All of the transfectants exhibited a similar dose response to mL-3 stimulation (not shown), and similar results were obtained in 3 separate experiments.

munoprecipitation of Shc or SHP-2 with β c, or vice versa, a possible interaction between Tyr577 and the Shc phosphotyrosine-binding domain has been reported.³⁷ It has been predicted that the N-terminal SH2 domain of SHP-2 binds to phosphotyrosines, followed by a β -branched residue at position +1 and a hydrophobic residue at +3, comprising a consensus sequence of Y-V/I/T-x-V/L/I.³⁸ In addition, involvement of residues that are N-terminal and more C-terminal to these residues, especially a hydrophobic residue (leucine, phenylalanine, or proline) at position +5 and valine, leucine, or glycine at position -2, in high-affinity binding has also been suggested.^{39,40} The sequence surrounding Tyr612 (L-G-Y612-L-C-L-P-A) reasonably matches these predictions, so there may be a direct interaction of this tyrosine with SHP-2. The mechanism by which the other 2 tyrosines, Tyr577 and Tyr695, facilitate SHP-2 phosphorylation still remains unknown.

Activation of STAT5 by GM-CSF was also seen to depend on β c tyrosines, but the pattern differed. The level of STAT5 phosphorylation was dramatically decreased by the lack of all the β c tyrosines (Fall mutant) but was restored by adding back any 1 (or 2, in the Y12 mutant) of the tyrosines, with each causing a different extent of recovery. Interestingly, the Y12 mutant induced markedly stronger phosphorylation of STAT5 than the Fall mutant, although these 2 mutants showed little transactivation of the β -casein promoter. Because STAT5 activity may be subject to regulation concerning serine phosphorylation,⁴¹ the Y12 mutant may not be able to transmit that signal. Studies concerning other cytokine receptors, such as the erythropoietin receptor and the IL-2 receptor β subunit, have shown that many of the tyrosines involved in STAT5 activation possess a leucine at position +3.³¹⁻³⁵ Consistent with this, 4 of the 8 β c tyrosines (Tyr612, Tyr750, Tyr806 and Tyr866) possess a

leucine at position +3, and these 4 tyrosines are all involved in STAT5 activation. However, STAT5 was also activated by the other β c tyrosines surrounded by relatively different motifs. It might be that these tyrosines recruit STAT5 in different ways, presumably through distinct adaptor proteins. Alternatively, the STAT5 SH2 domain could bind relatively divergent motifs, including those with and without a leucine at position +3.

We obtained evidence showing that the β c tyrosines play a critical role in transmitting growth-promoting signals, because the Fall mutant showed severely reduced stimulation of cell proliferation. This defect was restored, at least partly, by the addition of any 1 of the β c tyrosines. A dominant-negative STAT5 mutant inhibits, but does not completely suppress, IL-3-driven proliferation of BA/F3 cells, thereby suggesting an important role for STAT5 in mitogenic signaling.⁴² In addition, a recent study has reported that bone marrow-derived macrophages of STAT5A-null mice show defective GM-CSF-induced proliferation.⁴³ Taken together, these findings suggest that increased activation of STAT5 by any 1 β c tyrosine results in improved growth-promoting activity of the Y mutants. Although activation of STAT5 by the Y12 mutant was ambiguous as mentioned earlier, it may be that even a low level of STAT5 activation is sufficient to accelerate growth promotion although transactivation of the β -casein promoter requires a much higher level of STAT5 activation and/or additional signaling events. Alternatively, Tyr450/452 could stimulate proliferation by recruiting a molecule other than STAT5. It should be noted that the mutants Y3, Y4, and Y5 induced a relatively higher growth response than the other Y series mutants. There was a similar difference with respect to the induction of SHP-2 phosphorylation, Raf/ERK pathway activation, and c-fos transcription. Thus these signaling events,

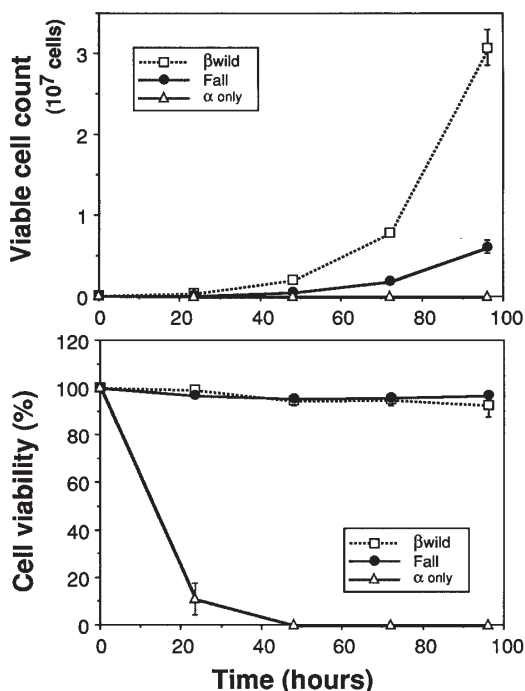


FIG 6. The Fall mutant supports survival of the BA/F3 transfectant. Long-term proliferation (upper panel) and the viability (lower panel) of BA/F3 transfectants that expressed the hGM-CSF receptor α subunit without β (α only), with wild-type β (β wild), or with the Fall mutant (Fall). Factor-depleted BA/F3 transfectants (1×10^5 cells) were seeded in the presence of hGM-CSF (10 ng/mL). Viable cell numbers were measured by trypan blue dye exclusion. All values are the average of 3 samples, and standard deviations are shown as error bars. Three independent stable clones with the Fall mutant gave similar results. All of the transfectants exhibited a similar growth response in the presence of mIL-3 instead of hGM-CSF, and all lost viability in the absence of any cytokine (not shown).

though not necessarily required for growth promotion, might have a role in inducing optimal cell proliferation under physiologic conditions.

GM-CSF supports not only the growth but also the survival of BA/F3 cells transfected with the GM-CSF receptor. We observed that BA/F3 cells expressing the Fall mutant continued proliferating at a lower growth rate and showed no significant loss of viability. In accordance with this, Fall was capable of suppressing factor depletion-induced apoptosis of BA/F3 cells in the presence of hGM-CSF (unpublished observation). Therefore the cell viability signal is transduced by β c independent of its tyrosine residues and hence is not likely to involve SHP-2, the Raf/ERK kinase cascade, or STAT5. Recent reports have revealed that protein kinase c-Akt (also known as protein kinase B), a downstream target of phosphoinositide 3'-OH kinase, mediates the antiapoptotic function of IL-3 by phosphorylating, and hence inactivating, the proapoptotic protein BAD.^{44,45} It should be of interest to examine whether the β c tyrosines are involved in regulating the phosphoinositide 3'-OH kinase/c-Akt/BAD pathway.

In conclusion, the present study demonstrated that β c tyrosine residues play a critical role in many, if not all,

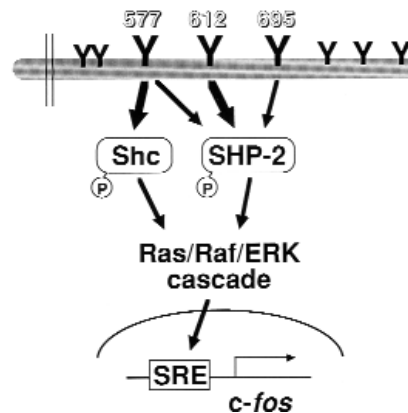


FIG 7. Proposed model of *c-fos* transcriptional activation by signaling from the hGM-CSF receptor. Y denotes β c tyrosine residues, and the numbers indicate their amino acid positions. The ligand and the receptor α subunit are omitted from this Figure.

aspects of GM-CSF signaling and functioning. These tyrosines each possess distinct, but overlapping, functions that activate multiple signaling pathways induced by GM-CSF. Further studies are required to determine the molecules that directly interact with the β c tyrosines and their roles in the β c-mediated signaling. These studies should shed more light on the molecular mechanisms by which GM-CSF regulates the proliferation, survival, and differentiation of hematopoietic cells.

REFERENCES

- Baldwin GC. The biology of granulocyte-macrophage colony-stimulating factor: effects on hematopoietic and nonhematopoietic cells. *Dev Biol* 1992;151:352-67.
- Gasson JC. Molecular physiology of granulocyte-macrophage colony-stimulating factor. *Blood* 1991;77:1131-45.
- Bazan JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA* 1990;87:6934-8.
- Gearing DP, King JA, Gough NM, Nicola NA. Expression cloning of a receptor for human granulocyte-macrophage colony-stimulating factor. *EMBO J* 1989;8:3667-76.
- Hayashida K, Kitamura T, Gorman DM, Arai K, Yokota T, Miyajima A. Molecular cloning of a second subunit of the receptor for human granulocyte-macrophage colony-stimulating factor (GM-CSF): reconstitution of a high-affinity GM-CSF receptor. *Proc Natl Acad Sci USA* 1990;87:9655-9.
- Kitamura T, Hayashida K, Sakamaki K, Yokota T, Arai K, Miyajima A. Reconstitution of functional receptors for human granulocyte/macrophage colony-stimulating factor (GM-CSF): evidence that the protein encoded by the AIC2B cDNA is a subunit of the murine GM-CSF receptor. *Proc Natl Acad Sci USA* 1991;88:5082-6.
- Miyajima A, Mui AL-F, Ogorochi T, Sakamaki K. Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. *Blood* 1993;82:1960-74.
- Duronio V, Clark-Lewis I, Federspiel B, Wieler JS, Schrader JW. Tyrosine phosphorylation of receptor β subunits and common substrates in response to interleukin-3 and granulocyte-macrophage colony-stimulating factor. *J Biol Chem* 1992;267:21856-63.
- Sakamaki K, Miyajima I, Kitamura T, Miyajima A. Critical cytoplasmic domains of the common β subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. *EMBO J* 1992;11:3541-9.
- McColl SR, DiPersio JF, Caon AC, Ho P, Naccache PH. Involvement of tyrosine kinases in the activation of human peripheral blood neutrophils

- by granulocyte-macrophage colony-stimulating factor. *Blood* 1991;78:1842-52.
11. Watanabe S, Muto A, Yokota T, Miyajima A, Arai K. Differential regulation of early response genes and cell proliferation through the human granulocyte macrophage colony-stimulating factor receptor: selective activation of the *c-fos* promoter by genistein. *Mol Biol Cell* 1993;4:983-92.
 12. Quelle FW, Sato N, Withuhn BA, Inhorn RC, Eder M, Miyajima A, et al. JAK2 associates with the β_c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. *Mol Cell Biol* 1994;14:4335-41.
 13. Watanabe S, Itoh T, Arai K. JAK2 is essential for activation of *c-fos* and *c-myc* promoters and cell proliferation through the human granulocyte-macrophage colony-stimulating factor receptor in BA/F3 cells. *J Biol Chem* 1996;271:12681-6.
 14. Zhao Y, Wagner F, Frank SJ, Kraft AS. The amino-terminal portion of the JAK2 protein kinase is necessary for binding and phosphorylation of the granulocyte-macrophage colony-stimulating factor receptor β_c chain. *J Biol Chem* 1995;270:13814-8.
 15. Itoh T, Muto A, Watanabe S, Miyajima A, Yokota T, Arai K. Granulocyte-macrophage colony-stimulating factor provokes RAS activation and transcription of *c-fos* through different modes of signaling. *J Biol Chem* 1996;271:7587-92.
 16. Sato N, Sakamaki K, Terada N, Arai K, Miyajima A. Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common β subunit responsible for different signaling. *EMBO J* 1993;12:4181-9.
 17. Welham MJ, Dechert U, Leslie KB, Jirik F, Schrader JW. Interleukin (IL)-3 and granulocyte/macrophage colony-stimulating factor, but not IL-4, induces tyrosine phosphorylation, activation, and association of SHPTP2 with Grb2 and phosphatidylinositol 3'-kinase. *J Biol Chem* 1994;269:23764-8.
 18. Matsuguchi T, Inhorn RC, Carlesso N, Xu G, Druker B, Griffin JD. Tyrosine phosphorylation of p95^{Nov} in myeloid cells is regulated by GM-CSF, IL-3 and steel factor and is constitutively increased by p210^{BCR/ABL}. *EMBO J* 1995;14:257-65.
 19. Odai H, Sasaki K, Iwamatsu A, Hanazono Y, Tanaka T, Mitani K, et al. The proto-oncogene product c-Cbl becomes tyrosine phosphorylated by stimulation with GM-CSF or Epo and constitutively binds to the SH3 domain of Grb2/Ash in human hematopoietic cells. *J Biol Chem* 1995;270:10800-5.
 20. Mui AL, Wakao H, O'Farrell AM, Harada N, Miyajima A. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *EMBO J* 1995;14:1166-75.
 21. Miyajima A, Schreurs J, Otsu K, Kondo A, Arai K, Maeda S. Use of the silkworm, *Bombyx mori*, and an insect baculovirus vector for high-level expression and secretion of biologically active mouse interleukin-3. *Gene* 1987;58:273-81.
 22. Watanabe Y, Kitamura T, Hayashida K, Miyajima A. Monoclonal antibody against the common β subunit (β_c) of the human interleukin-3 (IL-3), IL-5, and granulocyte-macrophage colony-stimulating factor receptors shows upregulation of β_c by IL-1 and tumor necrosis factor- α . *Blood* 1992;80:2215-20.
 23. Watanabe S, Mui AL-F, Muto A, Chen JX, Hayashida K, Yokota T, et al. Reconstituted human granulocyte-macrophage colony-stimulating factor receptor transduces growth-promoting signals in mouse NIH 3T3 cells: comparison with signalling in BA/F3 pro-B cells. *Mol Cell Biol* 1993;13:1440-8.
 24. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. Current protocols in molecular biology. New York: John Wiley & Sons, Inc; 1989.
 25. Mosmann T. Rapid colorimetric assays for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
 26. Watanabe S, Kubota H, Sakamoto KM, Arai K. Characterization of *cis*-acting sequences and *trans*-acting signals regulating early growth response 1 and *c-fos* promoters through the granulocyte-macrophage colony-stimulating factor receptor in BA/F3 cells. *Blood* 1997;89:1197-206.
 27. Durstin M, Inhorn RC, Griffin JD. Tyrosine phosphorylation of Shc is not required for proliferation or viability signaling by granulocyte-macrophage colony stimulating factor in hematopoietic cell lines. *J Immunol* 1996;157:534-40.
 28. Su B, Karin M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol* 1996;8:402-11.
 29. Darnell JE Jr. STATs and gene regulation. *Science* 1997;277:1630-5.
 30. O'Shea JJ. Jaks, STATs, cytokine signal transduction, and immunoregulation: Are we there yet? [published erratum appears in *Immunity* 1997;7:444]. *Immunity* 1997;7:1-11.
 31. Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* 1995;2:331-9.
 32. Damen JE, Wakao H, Miyajima A, Krosi J, Humphries RK, Cutler RL, et al. Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin-induced cell proliferation and Stat5 activation. *EMBO J* 1995;14:5557-68.
 33. Quelle FW, Wang D, Nosaka T, Thierfelder WE, Stravopodis D, Weinstein Y, et al. Erythropoietin induces activation of Stat5 through association with specific tyrosines on the receptor that are not required for a mitogenic response. *Mol Cell Biol* 1996;16:1622-31.
 34. Gobert S, Chretien S, Gouilleux F, Muller O, Pallard C, Dusanter-Fourt I, et al. Identification of tyrosine residues within the intracellular domain of the erythropoietin receptor crucial for STAT5 activation. *EMBO J* 1996;15:2434-41.
 35. Klingmuller U, Bergelson S, Hsiao JG, Lodish HF. Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5. *Proc Natl Acad Sci USA* 1996;93:8324-8.
 36. Hansen LH, Wang X, Kopchick JJ, Bouchelouche P, Nielsen JH, Galsgaard ED, et al. Identification of tyrosine residues in the intracellular domain of the growth hormone receptor required for transcriptional signaling and Stat5 activation. *J Biol Chem* 1996;271:12669-73.
 37. Pratt JC, Weiss M, Sieff CA, Shoelson SE, Burakoff SJ, Ravichandran KS. Evidence for a physical association between the Shc-PTB domain and the beta c chain of the granulocyte-macrophage colony-stimulating factor receptor. *J Biol Chem* 1996;271:12137-40.
 38. Case RD, Piccione E, Wolf G, Benett AM, Lechleider RJ, Neel BG, et al. SH-PTP2/Syp SH2 domain binding specificity is defined by direct interactions with platelet-derived growth factor beta-receptor, epidermal growth factor receptor, and insulin receptor substrate-1-derived phosphopeptides. *J Biol Chem* 1994;269:10467-74.
 39. Huyer G, Li ZM, Adam M, Huckle WR, Ramachandran C. Direct determination of the sequence recognition requirements of the SH2 domains of SH-PTP2. *Biochemistry* 1995;34:1040-9.
 40. Lee CH, Kominos D, Jacques S, Margolis B, Schlessinger J, Shoelson SE, et al. Crystal structures of peptide complexes of the amino-terminal SH2 domain of the Syp tyrosine phosphatase. *Structure* 1994;2:423-38.
 41. Beadling C, Ng J, Babbage JW, Cantrell DA. Interleukin-2 activation of STAT5 requires the convergent action of tyrosine kinases and a serine/threonine kinase pathway distinct from the Raf1/ERK2 MAP kinase pathway. *EMBO J* 1996;15:1902-13.
 42. Mui AL, Wakao H, Kinoshita T, Kitamura T, Miyajima A. Suppression of interleukin-3-induced gene expression by a C-terminal truncated Stat5: role of Stat5 in proliferation. *EMBO J* 1996;15:2425-33.
 43. Feldman GM, Rosenthal LA, Liu X, Hayes MP, Wynshaw-Boris A, Leonard WJ, et al. STAT5A-deficient mice demonstrate a defect in granulocyte-macrophage colony-stimulating factor-induced proliferation and gene expression. *Blood* 1997;90:1768-76.
 44. del Peso L, Gonzalez-Garcia M, Page C, Herrera R, Nunez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997;278:687-9.
 45. Songyang Z, Baltimore D, Cantley LC, Kaplan DR, Franke TF. Interleukin 3-dependent survival by the Akt protein kinase. *Proc Natl Acad Sci USA* 1997;94:11345-50.