Granulocyte-macrophage Colony-stimulating Factor Provokes RAS Activation and Transcription of c-fos through Different Modes of Signaling*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) provokes a proliferative response and induction of early-response genes such as c-fos in target cells. It also induces rapid tyrosine phosphorylation of cellular proteins, including the β subunit (βc) of its functional receptor. However, locations and functions of phosphorylated tyrosine residues within the βc are unclear. To elucidate the mechanism of the human GM-CSF receptor signal transduction, mutational analyses were made of the cytoplasmic domain of the βc, using murine BA/F3 cells. Deletion of the conserved box 1 motif resulted in loss of tyrosine phosphorylation of the βc, thereby indicating an essential role for this motif in activating the tyrosine kinase which phosphorylates βc. A C-terminal truncated mutant at position 589 activated the c-fos promoter, and this activation was diminished by a substitution at tyrosine 577 (Tyr	extsuperscript{577}). However, the same substitution in the full-length βc did not completely abrogate the c-fos promoter activation, hence, redundant signaling pathways probably exist. When we analyzed signaling molecules functioning downstream of the βc we found that Tyr	extsuperscript{577} is essential for Shc phosphorylation, while tyrosine phosphorylation of PTP1D was mediated through Tyr	extsuperscript{577} as well as through other site(s). We suggest that GM-CSF stimulates at least two modes of signals leading to ras activation, an event which ultimately gives rise to promoter activation of c-fos.

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a multifunctional cytokine which supports survival and proliferation of hematopoietic stem cells or progenitor cells, and also enhances multiple functions of mature neutrophils, macrophages, and eosinophils (1, 2). A functional high-affinity GM-CSF receptor (GM-CSFR) complex is composed of the ligand-specific α subunit(s) and the shared β subunits (βc), both belonging to the type I cytokine receptor superfamily (also known as hematopoietin receptor family) (3–5). The cytoplasmic domains of both the α and the βc are essential for GM-CSF-induced signaling; however, the βc has a relatively large cytoplasmic domain composed of about 430 amino acid residues (aa) (4, 6) and is likely to play pivotal roles in signal transduction.

Although the α and βc subunits do not contain characteristic motifs of kinase or phosphatase, GM-CSF does induce a rapid and reversible tyrosine phosphorylation of various cellular proteins which seem to be critical for biological functions; as revealed by experiments using tyrosine kinase inhibitors (6–8). AK2, a member of the Janus kinase family, is associated with the membrane-proximal region of the human (h) βc and is activated by hGM-CSF stimulation (9). Lyn and Fes/Fps are also activated (10, 11), but their roles in signaling remain unknown. One substrate for GM-CSF-activated tyrosine kinases is βc (4, 12). The locations of phosphorylated tyrosine residues and their functional significance require further study.

Previous studies done using a series of truncated mutants from the C terminus revealed that the cytoplasmic domain of βc contains two functional regions required for signaling (6, 8, 13). The membrane-proximal region, which contains the conserved box 1 motif, is involved in a proliferation signal and induction of c-myc mRNA. The distal region is essential for activation of the cascade of events involving Shc, Ras, Raf, mitogen-activated protein kinase, and for induction of the c-fos and c-jun mRNAs. Shc is an adaptor protein which contains the SH2 (Src homology 2) domain and the phosphotyrosine interaction domain (or phosphotyrosine-binding domain) (14–16) and is associated with phosphotyrosine residues of several growth factor receptors being phosphorylated, binding to Grb2, which in turn activates Ras by recruiting the Ras-guanine nucleotide exchange factor Sos (17, 18). Tyrosine phosphorylation of PTP1D (also known as Sh-PTP2 or Syp), a cellular protein tyrosine phosphatase which contains two SH2 domains (19–22), also results in its association with Grb2 and is therefore thought to induce the activation of Ras (22, 23). It has been reported that GM-CSF induces tyrosine phosphorylation of PTP1D and its association with Grb2 and the p85 subunit of phosphatidylinositol 3’-kinase (24). The functional region of the GM-CSFR responsible for this event is unknown.

To fully understand the mechanism of signal transduction of hGM-CSFR, we analyzed the signaling potential of various βc mutants by reconstituting high-affinity receptors in combination with the wild-type α subunit in murine interleukin-3 (mIL-3)-dependent BA/F3 cells. We found that tyrosine at position 577 (Tyr	extsuperscript{577}) of βc is important for activation of the c-fos pro-
GM-CSF Receptor Signal Transduction

MATERIALS AND METHODS

Cells and Culture—A mL3-dependent pro-B cell line, BA/F3, was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.25 mg/ml fetal calf serum. A mutant hGM-CSF producing in Escherichia coli was provided by Schering-Plough Corp. The mL3 expression in silkworm (Bombyx mori) was purified as described previously (25).

Antibodies—Rat monoclonal antibodies against the human common μc, 5A5, were prepared as described previously (26). Rabbit polyclonal antibodies against the extracellular domain of the human μc were obtained from Medical & Biological Laboratory Co. Ltd. (Nagoya, Japan). Anti-phosphotyrosine monoclonal antibodies (4G10) and antisera against Shc were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY), and anti-sera against PTP1D from Santa Cruz Biotechnologies (Santa Cruz, CA).

Plasmids—The hGM-CSFR α and βc (KH97) cDNAs were originally cloned into pCEV4 and pME18S vectors, respectively, as described previously. Promotor-luciferase fusion constructs contain the human c-fos promoter fragment (-404 to -41) fused to the luciferase fragment and was constructed as described elsewhere (27).

Results—Cells suspended in 200 μl of ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.5) (2 × 10⁵ cells) were mixed with 15 μg of plasmid DNA. Electroporation was then carried out using a Gene Pulser (Bio-Rad) set at 250 microfarads and 300 V. Transfectants were recovered and maintained in complete medium for 3 h. Viability cells were counted and aliquots were put into 96-well plates (3 × 10⁴ cells in 100 μl/well) in complete medium. Following 2 days of culture, G418 was added at a concentration of 1 mg/ml and the cells were cultured for 10 days. Surface expression of the transfected μc mutants or control vector, were used.

Plasmid DNAs were transiently transfected into BA/F3 cells using the DEAE-dextran method (27). For transient transfection, 3 μg of c-fos-luciferase fusion plasmid and 5 μg of the wild-type hGM-CSFR α cDNA plasmid, in combination with 5 μg of various βc mutants or control vector, were used. Three million cells were washed twice with TBS(+) (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄, 0.49 mM MgcL₂, 68 mM CaCl₂) and then mixed with DNA and DEAE-dextran (0.5 mg/ml) in 4 ml of TBS(+). Following a 30-min incubation at room temperature, cells were washed and cultured in complete media for 12 h, and then they were separated into three aliquots. After factor depletion for 6 h in a mL3-free medium containing 10% fetal calf serum, the cells were stimulated with hGM-CSF (5 ng/ml), mL3 (3 ng/ml), or no cytokine for 6 h, then harvested and used for luciferase assay. Proteins were extracted from cells by three cycles of freezing and thawing. Luciferase activity was measured using a luminometer (model LB9501; Berthold Lumat Co. Ltd., Japan). Protein concentration was determined using the BCA protein assay reagent (Pierce) according to the manufacturer’s instruction.

RESULTS

The Membrane-proximal Box 1 Motif Is Essential to Transduce Proliferation Signals—The membrane-proximal region (aa 456–517), which is required for the proliferation signal and activation of the c-myc gene, contains box 1 (from Trp382 to Pro405), and is adjacent to box 2 (from Val418 to Cys430). These motifs are conserved in the membrane-proximal regions of several type I cytokine receptors and have been shown to play critical roles in signaling events (31). We asked whether boxes 1 and 2 of the GM-CSFR are also important for signaling and for this, internal deletion mutants of these motifs, Δbox1, Δbox2, were used, respectively (Fig. 1). To determine their functions in supporting short-term proliferation, BA/F3 transfectants expressing the wild-type βc (αwild), Δbox1, or Δbox2, in combination with the wild-type α subunit were cultured for 24 h in the presence of various concentrations of hGM-CSF and MTT assays were performed. Cells expressing either αwild or Δbox2 proliferated in medium containing 1 ng/ml hGM-CSF, although the latter did show a slightly reduced sensitivity to hGM-CSF (Fig. 2, A and C). In contrast, Δbox1 at the same concentration of hGM-CSF did not transmit short-term proliferation signals (Fig. 2B). The response observed at a high concentration (100 ng/ml) was due to endogenous mouse βc (AIC2B), which stimulates cell proliferation in combination with the human α subunit (5). These results suggest that box 1, but not box 2, is essential to support hGM-CSF-induced short-term proliferation.

Box 1 is also essential for c-fos Promoter Activation Signals—Previous studies demonstrated that the membrane-distal region of the βc is required for the hGM-CSF-dependent induction of the c-fos mRNA (8, 13). However, these studies were carried out by using the βc mutants truncated from the C terminus, and it was unclear if the distal region alone was...
sufficient to transduce signals. We examined effects of the deletion of either box 1 or box 2 on the potential to activate the c-fos promoter by means of the transient transfection assay, using a reporter plasmid. The reporter plasmid carrying the c-fos promoter fused to the luciferase gene was co-transfected with expression constructs encoding for both the wild-type α subunit and a series of β mutants. After stimulation with cytokine, cell lysates were prepared and the luciferase activities were determined. The β wild activated the c-fos promoter in response to hGM-CSF (Fig. 3). However, Δbox1 did not activate the c-fos promoter even though it retained an intact distal region. Deletion of the box 2 motif resulted in a partial loss of the potential to activate the c-fos promoter, which means that box 2 is involved in, but is not essential for this signaling. Therefore, the distal region alone is insufficient for and the membrane-proximal region, especially box 1, is also required for c-fos promoter activation signals.

Box 1 Is Required for Activation of the Tyrosine Kinase Which Phosphorylates the βc—We next examined whether or not box 1 is involved in tyrosine phosphorylation of the βc. Stable transfectants expressing hGM-CSFR composed of the wild-type α and the βc mutant, were either left unstimulated or stimulated with hGM-CSF, and the immunoprecipitation was performed followed by Western blot analysis. As shown in Fig. 4A, the βc appeared in duplicated bands due to differences in glycosylation, since only a single band with a molecular mass (in agreement with the value predicted from the amino acid sequence of the βc) was detected after treatment with tunicamycin, the specific inhibitor of N-linked glycosylation. Blotting with anti-phosphotyrosine antibody (4G10) revealed that the Δbox1 mutant was not phosphorylated following hGM-CSF stimulation. In contrast, Δbox2 induced hGM-CSF-dependent tyrosine phosphorylation of the βc. These results indicate that box 1, but not box 2, is essential for tyrosine phosphorylation of the βc, and that this motif is important for activating tyrosine kinase phosphorylating the βc.

Within the Cytoplasmic Domain of the βc Is Important for Signaling to Activate the c-fos Promoter—To delineate the essential residue(s) or motif(s) for signaling leading to the c-fos promoter activation, we examined the potential of previous βc mutants as well as newly constructed ones (Fig. 1) to activate the c-fos promoter by the transient transfection assay. As shown in Fig. 3, β589, possessing only about 140 aa of the cytoplasmic domain, was still capable of activating the promoter at a level comparable to that seen with the β wild. However, the potential of the βc to activate the c-fos promoter was markedly reduced by truncation at aa 544. These data demonstrate requirement of the subregion covering aa 544 and 589 for this signaling event. These results, however, are inconsistent with other data that β626 is incapable of inducing the c-fos mRNA (8, 13). The cDNA construct for β626 was confirmed to be correct. However, we observed that the BaF2/β626 clone used in previous studies expresses a protein which does not match the expected size, only a smaller one. The subregion covering aa 544 and 589 contains one tyrosine

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2 A. Muto, unpublished data.

3 T. Itoh, unpublished data.
complexes with anti-NG/ml hGM-CSF for 10 min at 37°C. Cells were lysed and immune residue at position 577 (Tyr577). In the case of growth factor requirement of Tyr577 for signaling by using mutants in which Tyr577 was substituted for by phenylalanine. The level of the phosphotyrosine monoclonal antibody 4G10 (\(\alpha\)PTP1D, and there was a slight shift in mobility. Stimulation of cells expressing \(\beta\)589 also resulted in the same pattern of phosphorylation as observed in the full-length \(\beta\)c (Fig. 5A, lower panel). However, phosphorylation of this protein did not occur in cells expressing \(\beta\)544 or \(\beta\)wild; Y577F. Such being the case, Tyr577 is likely to be essential for tyrosine phosphorylation of Shc and is probably the critical site for Shc-mediated Ras activation.

Tyrosine Phosphorylation of PTP1D Is Mediated by Tyr577 as Well as by Other Functional Sites of the \(\beta\)c—As indicated above, the full-length \(\beta\)c containing a single substitution at Tyr577 is unable to induce tyrosine phosphorylation of Shc yet activates the c-fos promoter. This implies the existence of Shc-independent signaling pathways leading to activation of the c-fos promoter. Several lines of evidence strongly suggest that PTP1D functions as a positive regulator in growth factor receptor signaling, via Ras (22, 23, 32, 33). We next examined \(\beta\)c mutant-induced tyrosine phosphorylation of PTP1D.

Immunoprecipitates obtained using an anti-PTP1D antibody from BA/F3 transfectants were analyzed by Western blotting. As shown in Fig. 5B, exposure of hGM-CSF to cells expressing \(\beta\)wild led to the induction of tyrosine phosphorylation of PTP1D, and there was a slight shift in mobility. Stimulation of cells expressing \(\beta\)589 also resulted in the same pattern of phosphorylation as observed in the full-length \(\beta\)c (Fig. 5B, lower panel). However, \(\beta\)wild; Y577F still induced tyrosine phosphorylation of PTP1D and co-immunoprecipitated proteins. Thus, while Tyr577 is involved in tyrosine phosphorylation of PTP1D, unlike Shc, it is not essential for this event. The activation of PTP1D

![Figure 3](http://www.jbc.org/) GM-CSF Receptor Signal Transduction

**FIG. 3.** Potential of \(\beta\)c mutants to activate the c-fos promoter. Activation of c-fos promoter by each mutant \(\beta\)c was measured by the transient transfection assay using the c-fos promoter-luciferase fusion construct as a reporter gene. Continuously growing BA/F3 cells were transfected with plasmids containing the wild-type hGM-CSFR \(\alpha\) subunit cDNA and those containing each of the \(\beta\)c mutants together with the c-fos promoter-luciferase reporter plasmid, as described under "Materials and Methods." After 6 h factor depletion, cells were either left unstimulated or stimulated with 5 ng/ml hGM-CSF or 1 ng/ml mIL-3 for 6 h. Cell lysates were prepared and subjected to the luciferase assay. The c-fos promoter activity was calculated by dividing the luminescence intensity (relative light units per microgram of total protein) of no stimulation or hGM-CSF stimulation by that of mIL-3 stimulation, and are presented as a percentage of that of \(\beta\)wild. All values are the average of at least two experiments and standard deviations are shown as error bars.

**FIG. 4.** Tyrosine phosphorylation of the \(\beta\)c. The factor-deprived BA/F3 transfectants (1 \(\times\) 107 cells each) stably expressing either the wild-type \(\beta\)c (A and B), \(\Delta\)box1, \(\Delta\)box2 (A), \(\beta\)589, \(\beta\)544, \(\beta\)wild; Y577F, or \(\beta\)589; Y577F (B) were either left unstimulated or stimulated with 10 ng/ml hGM-CSF for 10 min at 37°C. Cells were lysed and immune complexes with anti-\(\beta\)c monoclonal antibody 5A5 were precipitated. Protein samples were separated by SDS-7.5% polyacrylamide gel electrophoresis and Western blot analyses were performed using anti-phosphotyrosine monoclonal antibody 4G10 (left column) or anti-\(\beta\)c polyclonal antibodies (right column).

Tyr577 in full-length \(\beta\)c (\(\beta\)wild; Y577F) did not significantly impair the function of the receptor. These data demonstrated that: 1) Tyr577 is an essential residue for activation of the c-fos promoter, at least in \(\beta\)589, and 2) other sites located C-terminal to aa 589 in the full-length \(\beta\)c play a similar, if not identical, role.

Tyr577 of the \(\beta\)c Is Phosphorylated following Ligand Stimulation—As it seemed clear that Tyr577 is involved in signaling from the \(\beta\)c, we next examined whether or not this tyrosine residue is actually the target site for the hGM-CSF-dependent phosphorylation and here we used immunoprecipitation and Western blot analysis. As shown in Fig. 4B, hGM-CSF induces tyrosine phosphorylation of either \(\beta\)589 or \(\beta\)wild, but not \(\beta\)544 or Tyr577-mutated \(\beta\)589. Since Tyr577 is the only tyrosine residue within the region between aa 545 and 589, these results show that Tyr577 is a target site of phosphorylation. However, \(\beta\)wild; Y577F, the full-length \(\beta\)c with a single substitution of phenylalanine for Tyr577 was still phosphorylated following ligand stimulation. Therefore, hGM-CSF induces phosphorylation of multiple tyrosine residues in the \(\beta\)c, including Tyr577. This result is consistent with findings that \(\beta\)wild; Y577F is capable of activating the c-fos promoter.

Tyr577 of the \(\beta\)c Is Required for Tyrosine Phosphorylation of Shc—Next, we investigated the nature of the signaling molecules functioning downstream of the phosphorylated \(\beta\)c. Our previous study demonstrated that the hGM-CSF induces tyrosine phosphorylation of Shc through the membrane-distal region of the \(\beta\)c, therefore, we searched for the possible involvement of Tyr577 for activation of Shc.

Cell lysates were prepared from either unstimulated or hGM-CSF-stimulated BA/F3 transfectants. Immunoprecipitations were performed using the anti-Shc antibody, and the proteins precipitated were subjected to Western blot analysis. This antibody immunoprecipitated 46- and 52-kDa species of Shc proteins (p46\(^{\text{Shc}}\) and p52\(^{\text{Shc}}\), respectively) from BA/F3 transfectants (Fig. 5A, lower panel). Blotting with an antiphosphotyrosine antibody revealed that p46\(^{\text{Shc}}\) was phosphorylated in a constitutive manner. \(\beta\)wild and \(\beta\)589 apparently induced tyrosine phosphorylation of p52\(^{\text{Shc}}\) in a ligand-dependent manner (Fig. 5A, upper panel). However, phosphorylation of this protein did not occur in cells expressing \(\beta\)544 or \(\beta\)wild; Y577F. Such being the case, Tyr577 is likely to be essential for tyrosine phosphorylation of Shc and is probably the critical site for Shc-mediated Ras activation.
is mediated by Tyr\textsuperscript{577} as well as by other functional sites located at position C-terminal to aa 589.

**DISCUSSION**

We analyzed functional residues of the cytoplasmic domain of the βc, and found that: 1) the box 1 motif is essential for activation of the tyrosine kinase which phosphorylates βc and 2) Tyr\textsuperscript{577} is one phosphorylation site within the βc and is critical for the activation of Shc (Fig. 6). Our results also suggest that GM-CSF stimulates at least two signaling pathways, one is mediated by Shc and the other by PTP1D, both leading to activation of Ras and ultimately to transcriptional activation of the c-fos gene.

In contrast to box 1, the box 2 motif of the βc is dispensable for signaling, the Δbox2 mutant was capable of stimulating short-term proliferation. However, cells expressing Δbox2 showed a slightly reduced sensitivity to hGM-CSF, and the level of the c-fos promoter activation by Δbox2 was about half that by the βc wild. Therefore, box 2 of the βc appears to enhance activity of the receptor. It should be noted that in other type I cytokine receptors, such as the interleukin-2 receptor β subunit, gp130, and the erythropoietin receptor, box 2 is essential for cell proliferation and other signaling events (31, 35, 36). The molecular basis for this discrepancy and functions of box 2 will need further attention.

We also showed that Tyr\textsuperscript{577} within the cytoplasmic domain of the βc is phosphorylated following ligand stimulation, and is crucial for hGM-CSF-dependent signal transduction. Substitution at Tyr\textsuperscript{577} abrogated the signals leading to activation of the c-fos promoter by βs98, and reduced those by the full-length βc. However, as βwild;Y577F partially activated the c-fos promoter means that other active site(s) located at position(s) C-terminal to 589 should be considered.

**Fig. 6.** A putative model for signal transduction of GM-CSFR.

The βc subunit of the hGM-CSFR contains the conserved box 1 and box 2 motifs, and we found that box 1 has a critical role in hGM-CSF-induced signaling. The Δbox1 mutant failed to induce short-term proliferation in response to hGM-CSF. Box 1 also proved to be essential for activation of the c-myc promoter in the transient transfection assay using the c-myc-CAT reporter plasmid.\textsuperscript{4} In addition to these signaling events, box 1 is essential for c-fos promoter activation signals for which only the membrane-distal region of the βc has heretofore thought to be required. The Δbox1 mutant did not induce tyrosine phosphorylation of the βc following hGM-CSF stimulation. This indicates that box 1 is required for activation of some tyrosine kinase which phosphorylates βc. The requirement of box 1 for c-fos promoter activation seems reasonable since tyrosine phosphorylation of the βc plays a role in signaling from the distal region. However, the molecular nature of the tyrosine kinase which phosphorylates the βc will need to be clarified. Recently, it was reported that box 1 of the βc directly interacts with the N-terminal portion of JAK2 (34). We also found that box 1 is necessary for activation of JAK2 and that overexpression of both JAK2 and the βc in COS7 cells results in tyrosine phosphorylation of the βc.\textsuperscript{4} Thus, it is possible, although not yet proven, that JAK2 phosphorylates the tyrosine residues of the βc.

\textsuperscript{4} S. Watanabe, unpublished data.

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**Fig. 5.** Tyrosine phosphorylation of SH2-containing proteins in BA/F3 transfectants. The factor-deprived BA/F3 transfectants (5 × 10\textsuperscript{6} cells each) were either left unstimulated or stimulated with 10 ng/ml hGM-CSF for 10 min at 37°C. Cells were lysed and immune complexes with anti-Shc antibody (A) or anti-PTP1D antibody (B) were precipitated. Protein samples were separated by SDS-7.5% polyacrylamide gel electrophoresis and tyrosine-phosphorylated proteins were identified by Western blot using anti-phosphotyrosine antibodies (upper panels). Immunoprecipitated Shc or PTP1D proteins were identified by blotting with anti-Shc antibodies or anti-PTP1D antibodies (lower panels), respectively. Molecular size standards are shown in kDa on the left. The positions of p52\textsuperscript{shc} and p46\textsuperscript{shc} (A) and PTP1D (B) are shown on the right by arrows.
to aa S89 perform a similar, if not identical, function and that either Tyr$^\gamma 77$ or the other site(s) alone is sufficient to transduce signals. Since tyrosine phosphorylation of the $\beta c$ occurs at multiple sites, including Tyr$^\gamma 77$, phosphorylated tyrosine(s) other than Tyr$^\gamma 77$ are likely to have a role in signaling.

Previous studies have shown that both induction of the c-fos/c-jun mRNAs and activation of the Ras/Raf/mitogen-activated protein kinase cascade by hGM-CSF depend on the membrane-distal domain, thereby implying that these events are related (8, 13). Ras is indeed required for induction of the c-fos mRNA, as the dominant-negative Ras mutant completely inhibited the GM-CSF-dependent activation of the c-fos promoter via the wild-type $\beta c$ in the transient transfection assay. Thus, it appears that the signaling pathways originating from both Tyr$^\gamma 77$ and other functional sites independent of Tyr$^\gamma 77$ merge upstream of Ras. Both Shc and PTP1D, when tyrosine phosphorylated, are known to associate with Grb2 and subsequently activate Ras. Our data concerning activation of these molecules strongly suggest that hGM-CSF stimulates at least two independent molecular events leading to Ras activation, and that residues of the $\beta c$ responsible for these events are different although overlapping. As tyrosine phosphorylation of Shc was abrogated by the single substitution at Tyr$^577$ of the full-length $\beta c$, Tyr$^\gamma 77$ has an essential role in Shc activation. It has been reported that Shc protein associates with the phosphorylated $\beta c$ and that this association is mediated through its SH2 domain (37). Although co-immunoprecipitating Shc with the $\beta c$ or vice versa has yet to be done, it is of considerable interest as to whether or not Shc directly binds to phosphorylated Tyr$^77$. In contrast to Shc, both $\beta S89$ and $\beta WY77$ are capable of inducing tyrosine phosphorylation of PTP1D, indicating that activation of PTP1D is mediated by multiple sites, including Tyr$^\gamma 77$. Since PTP1D contains two SH2 domains, it may be that this molecule associates with the receptor in such a manner that each SH2 domain interacts, either directly or indirectly, with distinct phosphotyrosine residue of the $\beta c$, one of which is sufficient for signaling. The N-terminal SH2 domain of PTP1D was predicted to bind to a consensus sequence of Tyr-Val-Xaa-Val/Ile/Leu/Pro with low selectivity (38). The sequence surrounding Tyr$^\gamma 77$ (Tyr$^\gamma 77$-Leu-Gly-Pro) partially matches this sequence. There is no available evidence that PTP1D directly interacts with $\beta c$. It is noteworthy that $\beta S89$;Y77F slightly stimulates phosphorylation of PTP1D and this may partly account for the observation that $\beta S44$ or $\beta S89$;Y77F induces slightly higher levels of the c-fos promoter activation than seen with $\beta c$ or the vector control. One possible explanation for this weak phosphorylation is that activation of the tyrosine kinase which phosphorylates PTP1D depends on the region located N-terminal to aa S44, while efficient phosphorylation is mediated by Tyr$^77$ and other sites located more C-terminal by recruiting the molecule onto the receptor. This notion is given some support by the finding that the overexpression of both JAK2 and PTP1D in COS7 cells results in tyrosine phosphorylation of PTP1D.6

Our observations imply that tyrosine phosphorylation of the $\beta c$ is crucial for signal transduction of hGM-CSF. Based on this, we are now analyzing functional tyrosine residues of the $\beta c$ other than Tyr$^\gamma 77$ as well as their downstream signaling molecules. All these observations will help unravel mechanisms which regulate proliferation and differentiation of hematopoietic cells by hGM-CSF.

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