Evidence for a Physical Association between the Shc-PTB Domain and the $\beta_c$ Chain of the Granulocyte-Macrophage Colony-stimulating Factor Receptor*

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Granulocyte-macrophage colony-stimulating factor (GM-CSF) regulates the growth and function of several myeloid cell types at different stages of maturation. The effects of GM-CSF are mediated through a high affinity receptor that is composed of two chains: a unique, ligand-specific $\alpha$ chain and a $\beta$ common chain ($\beta_c$) that is also a component of the receptors for interleukin 3 (IL-3) and IL-5. $\beta_c$ plays an essential role in the transduction of extracellular signals to the nucleus through its recruitment of secondary messengers. Several downstream signaling events induced by GM-CSF stimulation have been described, including activation of tyrosine kinases and tyrosine phosphorylation of cellular proteins (including $\beta_c$) and activation of the Ras/mitogen-activated protein kinase and the JAK/STAT pathways. A region of the $\beta_c$ cytoplasmic tail (amino acids 517–763) has been reported to be necessary for tyrosine phosphorylation of the adapter protein, Shc, and for the subsequent GM-CSF-induced activation of Ras. In this paper, we describe a physical association between the tyrosine phosphorylated GM-CSF receptor (GMR)-$\beta_c$ chain and Shc in vivo. Using a series of cytoplasmic truncation mutants of $\beta_c$ and various mutant Shc proteins, we demonstrate that the N-terminal phosphotyrosine-binding (PTB) domain of Shc binds to a short region of $\beta_c$ (amino acids 549–656) that contains Tyr577. Addition of a specific phosphopeptide encoding amino acids surrounding this tyrosine inhibited the interaction between $\beta_c$ and Shc. Moreover, mutation of a key residue within the phosphotyrosine binding pocket of the Shc-PTB domain abrogated its association with $\beta_c$. These observations provide an explanation for the previously described requirement for Tyr577 of $\beta_c$ for GM-CSF-induced tyrosine phosphorylation of Shc and have implications for Ras activation through the GM-CSF, IL-3, and IL-5 receptors.

The biological effects of granulocyte-macrophage colony stimulating factor (GM-CSF) are mediated by the interaction of the cytokine with a high affinity receptor expressed on certain cell types of hematopoietic lineage. The GM-CSF receptor (GMR) is composed of an $\alpha$ and a $\beta$ chain. Expression of the $\alpha$ chain alone is sufficient for low affinity binding of ligand. The human $\beta$ chain does not directly bind ligand; however, its association with the $\alpha$ chain confers high affinity binding of the cytokine. The $\beta$ chain of GMR is referred to as $\beta_c$ because it also interacts with distinct $\alpha$ subunits of the IL-3 and IL-5 receptors to form heterodimeric high affinity receptors for IL-3 and IL-5, respectively (reviewed in Ref. 1). While the cytoplasmic domains of $\alpha$ and $\beta_c$ do not influence ligand binding, they are essential for transmitting the mitogenic signal induced by GM-CSF (2). Although $\beta_c$ has no intrinsic kinase activity, the cytoplasmic domain of $\beta_c$ is necessary for the activation of cytosolic tyrosine kinases, such as JAK2 (3), small GTP-binding proteins, such as Ras (4, 5), and for induction of c-fos, c-jun, and c-myc (6). Mutational analyses have identified regions of the $\beta_c$ cytoplasmic tail that are required for mediating specific downstream signaling events. Induction of c-myc and activation of JAK2 require the membrane-proximal 60 amino acids of the cytoplasmic tail of $\beta_c$, whereas a membrane distal domain, between amino acids 517 and 763, is necessary for Ras activation and the induction of c-fos and c-jun (3, 7, 8). A site within this latter domain has been implicated in the tyrosine phosphorylation of $\beta_c$. A specific point mutation at tyrosine 750 abolishes phosphorylation of $\beta_c$ (9, 10).

Shc is an adapter protein that has been implicated in linking growth factor, cytokine and antigen receptors to Ras signaling (reviewed in Ref. 11). Previous studies have demonstrated that Shc is phosphorylated on tyrosine following binding of GM-CSF, IL-3, and IL-5 to their receptors (7, 9, 10, 12). Phosphorylated Shc subsequently interacts with another adapter protein, Grb2, which, in turn, interacts with the GTP/GDP nucleotide exchange factor for Ras, mSOS (13, 14). Shc contains two domains capable of interacting with tyrosine-phosphorylated proteins: an N-terminal phosphotyrosine-binding (PTB) domain and a C-terminal SH2 domain (15–17). The interaction of Shc with tyrosine-phosphorylated receptors and the localization of the Shc-Grb2-mSOS complex at the membrane have been implicated in Ras activation. A region of $\beta_c$ (517–763) that is necessary for Ras activation is also required for Shc phosphorylation upon GM-CSF stimulation (7). A point mutation within $\beta_c$ that changes tyrosine 577 to phenylalanine abolished GM-CSF-induced phosphorylation of Shc and the association of Shc with Grb2 (8, 9).

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The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; GMR, GM-CSF receptor; IL, interleukin; PTB, phosphotyrosine-binding; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; HA, hemagglutinin; GST, glutathione S-transferase; EGFR, epidermal growth factor receptor.

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One hypothesis for the $\beta_c$ $\text{Tyr}^{577}$-dependent Shc phosphorylation during GM-CSF stimulation is that Shc interacts with phosphorylated $\beta_c$ $\text{Tyr}^{577}$ through its SH2 or PTB domain and this interaction leads to Shc phosphorylation. We have used a transient transfection system to address this question and show here a physical interaction between Shc and tyrosine-phosphorylated $\beta_c$. An analysis of the domains of these proteins that are critical for this interaction revealed that the region spanning amino acids 549–656 of $\beta_c$ interacts with the recently described PTB domain of Shc. Addition of a 16-residue phosphopeptide that encompasses $\text{Tyr}^{577}$ inhibited the interaction between $\beta_c$ and Shc. In addition, mutation of a key residue of the phosphotyrosine-binding pocket in the PTB domain of Shc abolished this interaction. These observations suggest that the Shc-PTB domain interacts with the phosphorylated $\beta_c$ chain and provide an explanation for the requirement for $\beta_c$ $\text{Tyr}^{577}$ in GM-CSF-dependent Shc phosphorylation.

MATERIALS AND METHODS

Antibodies—Anti-GST and anti-$\beta_c$ monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified anti-HA antibody (clone 12CA5) was purchased from Boehringer Mannheim. Horseradish peroxidase-conjugated anti-phosphotyrosine (RC20) antibody was obtained from Transduction Laboratories (Lexington, KY).

Cells and Transfections—COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FCS, 100 units/ml penicillin-streptomycin and 2 ml/l-glutamine. Cells were split on the day before transfection. Indicated concentrations of plasmid DNA were added to 7.5 ml of FCS/DMEM containing 100 $\mu$g/ml chloroquine and 400 $\mu$g/ml DEAE-dextran and incubated for 3.5 h at 37°C. Cells were treated with 10%DMF/PBS for 2 min at room temperature, washed once with PBS, and fresh 10%FCS/DMEM was added. On the following day, the medium was replaced with fresh 10%FCS/DMEM, and cells were cultured for an additional 18 h before use.

Plasmids—The pMT21 vector encoding wild type human GMR-$\alpha_2$, wild type GMR-$\beta_c$, and mutant GMR-$\beta_c$ constructs with an HA tag have been described previously (2). Full-length human Shc or partial regions of Shc were generated by the polymerase chain reaction and were expressed as GST fusion proteins by subcloning into the pEB eukaryotic expression vector (18, 19). The amino acid sequences encoded by the different Shc constructs were full-length (1–473), N (1–232), NCH (1–377), CH-2SH2 (233–473), CH (233–377). The point mutations within the PTB domain were generated by polymerase chain reaction as described previously (18). All constructs, including the presence of appropriate mutations, were confirmed by DNA sequencing. cDNAs encoding HA-tagged wild type and kinase-defective murine JAK2, cloned into pEF-BOS, were kindly provided by Dr. Dwayne Barber (Dana-Farber Cancer Institute, Boston, MA) (20).

Precipitations and Immunoblotting—Transfected cells were washed once with PBS and lysed in 0.5 ml of buffer (50 mM Tris, 0.5% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 10 mM Na3P, 0.4 mM Na2VO4, 10 $\mu$g/ml leupeptin and aprotinin, and 4 mM phenylmethylsulfonyl fluoride) for 15 min at 4°C. Lysates were collected and spun at 13,000 rpm for 10 min. Supernatants were transferred to a fresh tube and incubated with glutathione-Sepharose beads for 2 h at 4°C to precipitate the GST-tagged Shc proteins. Beads were washed four times, and the bound proteins were analyzed as described previously using the ECL detection system (Amersham Corp.) (21). In peptide inhibition studies, GST-Shc was precipitated from lysates of transfected cells and incubated with 100 $\mu$M control peptide derived from EGFR (sequence surrounding $\text{Tyr}^{1173}$, TAENAEYLRVA) or indicated concentrations of $\beta_c$ peptide (QASSDFNPGRPYGPH) for 30 min at 4°C, followed by addition of lysates from cells expressing $\beta_c$ and JAK2.

RESULTS AND DISCUSSION

Shc Interacts with the Phosphorylated $\beta_c$—Previous studies have demonstrated that Shc is phosphorylated following GM-CSF stimulation (12). Phosphorylated Shc subsequently interacts with Grb2 that, in turn, binds mSOS. It has been presumed that Shc interacts with the tyrosine phosphorylated $\beta_c$, and this interaction localizes the Shc-Grb2-mSOS complex to the membrane. However, a physical association of Shc with $\beta_c$ has not been clearly demonstrated. To determine if a direct interaction of Shc with $\beta_c$ occurs, we co-expressed $\beta_c$ with Shc and JAK2 in COS-7 cells. Specifically, we expected that the co-expression of JAK2 and $\beta_c$ might lead to the tyrosine phosphorylation of $\beta_c$ at relevant sites, allowing $\beta_c$ to interact with Shc. We assessed the tyrosine phosphorylation status of $\beta_c$ when expressed in COS-7 cells by anti-$\beta_c$ immunoprecipitation and anti-phosphotyrosine immunoblotting. When $\beta_c$ was co-expressed with Shc alone, there was no detectable phosphorylation of $\beta_c$ (Fig. 1A). In contrast, co-expression of JAK2 with $\beta_c$ led to significant tyrosine phosphorylation of $\beta_c$. Moreover, expression of $\beta_c$ with kinase-deficient JAK2 did not result in tyrosine phosphorylation of $\beta_c$ (Fig. 1C).

We next determined if phosphorylation of $\beta_c$ by JAK2 would lead to an association between $\beta_c$ and Shc. Proteins from cells co-transfected with $\beta_c$, JAK2, and GST-Shc were precipitated with glutathione-Sepharose beads, and proteins co-precipitating with GST-Shc were visualized by anti-phosphotyrosine immunoblotting. Co-expression of JAK2 and GST-Shc alone, or $\beta_c$ and GST-Shc alone, did not lead to an association of $\beta_c$ with Shc (Fig. 1B). In the presence of wild type JAK2, $\beta_c$ became phosphorylated and associated with co-expressed GST-Shc. However, the co-expression of a kinase-deficient JAK2 with $\beta_c$ and GST-Shc failed to lead to an association of $\beta_c$ with Shc, confirming the requirement for JAK2 kinase activity for this association. Anti-phosphotyrosine immunoblot analysis of total cell lysates from these transfectedants revealed that $\beta_c$ was not phosphorylated in the kinase-deficient JAK2 expressing cells (Fig. 1C), which may explain the lack of binding of $\beta_c$ to Shc. Thus, we could obtain JAK2-dependent tyrosine phosphorylation of $\beta_c$ and detect an interaction between $\beta_c$ and Shc.

To determine the region of $\beta_c$ necessary for the interaction with Shc, we expressed deletion mutants of $\beta_c$ (schematically shown in Fig. 2A) with JAK2 and GST-Shc as described above.

![Fig. 1](https://via.placeholder.com/150)
Deletion mutants of βc indicated that amino acids between 549 and 656 are essential for the association between βc and Shc (Fig. 2B). Previous studies based on site-directed mutagenesis of tyrosines within βc have indicated that Tyr577 is necessary for Shc phosphorylation and its subsequent interaction with Grb2 (9). Tyrosine 577 resides within the region (549–656) that we find essential for an interaction between phosphorylated βc and Shc. Immunoblotting with an anti-GST antibody confirmed that comparable amounts of GST-Shc were precipitated in each sample (Fig. 2C). In addition, immunoblot analysis of total cell lysates with an anti-HA antibody revealed that all the HA-tagged deletion mutants were expressed (Fig. 2D).

The PTB Domain of Shc Interacts with βc — The structure of the Shc adapter protein suggests that it could interact with βc through either its N-terminal PTB domain, C-terminal SH2 domain, or both. The Shc-SH2 domain interacts with phosphorylated transmembrane receptors, such as the EGF-R (15, 22), T cell receptor-ζ (23), and the platelet-derived growth factor receptor (24). The PTB domain is a newly characterized region that also interacts with tyrosine-phosphorylated proteins and has been shown to interact with receptors for EGF (17), nerve growth factor (25, 26) and IL-2 (27). The Shc-PTB domain (residues 17–207) is contained within the N terminus (residues 1–232) of Shc. We assessed the interaction of different domains of Shc with full-length βc. The various GST-Shc constructs are schematically represented in Fig. 3A. While Shc proteins that contained the N-terminal PTB domain (full-length Shc, NCH,

![Fig. 2. A region of the βc chain (amino acids 549–656) is essential for its interaction with Shc. A, schematic of βc deletion mutants with C-terminal HA tag. B, cells were transfected as described above and precipitations were performed using glutathione-Sepharose beads. Co-precipitation of βc with GST-Shc was visualized by immunoblotting with anti-phosphotyrosine antibody. C, membranes were stripped and immunoblot analysis of GST-Shc expression with anti-GST antibody was performed. D, total cell lysates were subjected to immunoblot analysis with anti-HA antibody to visualize the expression of βc mutants. The βc549 protein migrates just below a nonspecific band present in all lanes.](image)

![Fig. 3. The PTB domain of Shc is necessary and sufficient for interaction with βc. A, schematic of GST-Shc mutant constructs and summary of their interaction with βc. B, COS-7 cells were transfected and analyzed as described in Fig. 1 and “Materials and Methods.” Co-precipitation of βc with GST-Shc proteins using glutathione-Sepharose beads, was visualized by anti-phosphotyrosine immunoblotting. C, membranes were stripped and reprobed with anti-GST antibody to assess the expression of different GST-Shc proteins. D, cells were transfected with 0.5 μg of the indicated ShcN mutants along with 4 μg of JAK2 and 0.5 μg of βc656 as described. Lysates were subjected to precipitation with glutathione-Sepharose beads and analyzed by anti-phosphotyrosine immunoblotting. Reprobing the membrane with anti-GST antibody revealed comparable expression of different Shc proteins (data not shown).](image)
did not affect binding of GST-ShcN to β656. These results confirm that the physical association of Shc with β6 is dependent upon an intact phosphotyrosine-binding pocket within the PTB domain of Shc.

Recent studies have indicated that the PTB domain of Shc recognizes amino acid residues N-terminal to the phosphotyrosine and preferentially interacts with β-turn forming sequences containing a critical asparagine residue at the −3 position relative to the phosphotyrosine (16, 18, 28–31). The only tyrosine within the β6 cytoplasmic domain that fits such a tyrosine-based recognition motif is Tyr577 (SFDFNGPYPGLGP), which has been implicated in GM-CSF-dependent Shc phosphorylation. Our observations reported here demonstrate that the Shc-PTB domain interacts with tyrosine phosphorylated β6 through a region that encompasses Tyr577. To provide direct evidence that Shc interacts with β6 through Tyr577, we synthesized a phosphopeptide encoding Tyr577 (QASSFDFNGPYPGLGPPH) specifically inhibited the interaction of the Shc-β6 interaction. Increasing concentrations of peptide were incubated with GST-ShcN followed by the addition of the lysates from β656- and Jak2 expressing cells. In the absence of peptide, or the presence of 100 µM EGFR control peptide (previously shown not to inhibit the Shc-PTB interaction with phosphopeptides (28)), association of β6 with Shc was unaffected (Fig. 4). In contrast, a peptide encoding Tyr577 (QASSFDFNGPYPGLGPPH) specifically inhibited the interaction between β6 and Shc. Titration of the peptide indicated that 10 µM of the specific peptide significantly affected the association, while at 50 µM the association was completely disrupted. These observations are consistent with previously reported affinities of Shc-PTB for phosphopeptides (28, 29).

Although Tyr750 of β6 was originally implicated in Shc phosphorylation, it has subsequently been reported that this may be an indirect effect due to the failure to phosphorylate β6 (9, 10). This is consistent with our data that truncation mutants that lack Tyr750, but are phosphorylated at Tyr577, can still interact with Shc. Thus, we favor the hypothesis that recruitment of Shc to β6 occurs through Tyr577, which is further supported by our peptide inhibition studies.

Taked together, these data suggest a model whereby GM-CSF stimulation leads to recruitment of Shc to GMR first, followed by tyrosine phosphorylation of Shc. This situation is analogous to the IL-2Rβ chain, which shares a high degree of structural homology in its cytoplasmic tail with β6 of GMR. Interestingly, Shc also interacts with phosphorylated Tyr338 of IL-2Rγ through its PTB domain. Mutation of Tyr338 of the Shc binding site on the IL-2Rβ chain, failed to lead to Shc phosphorylation (27, 32). An intriguing possibility is that the interaction of the Shc-PTB domain with β6 or IL-2Rβ allows for the SH2 domain of Shc to recruit other phosphotyrosine-containing proteins (to the proximity of the receptor) that may also contribute to intracellular signaling. It remains to be established if this reflects a pattern of Shc-mediated signaling that is common to type I cytokine receptor superfamily members.