Oncostatin M Induces Association of Grb2 with Janus Kinase JAK2 in Multiple Myeloma Cells

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Summary

Oncostatin M (OSM) is a 28-kD glycoprotein recently identified as a growth factor for human multiple myeloma cells. It belongs to a family of distantly related cytokines that includes interleukin 6, ciliary neurotrophic factor, leukemia-inhibitory factor, and interleukin 11. These cytokines initiate signaling by inducing either homodimerization of gp130 or heterodimerization of gp130 with leukemia-inhibitory factor receptor β components. Such dimerization in turn activates receptor-associated tyrosine kinases. In the present study using U266B1 human multiple myeloma cells, we show that OSM induces tyrosine phosphorylation and activation of JAK2, but not JAK1 or Tyk2, kinases. The results also demonstrate that OSM induces direct interaction of JAK2 kinase with Grb2, an SH2/SH3 domain containing adaptor protein. The SH2 domain of Grb2 is directly associated with tyrosine-phosphorylated JAK2. Furthermore, the presence of Sos in the JAK2–Grb2 complex suggests a role for Ras in OSM-transduced signaling.

Oncostatin M (OSM),[1] a mediator of pleiotropic biological activities, binds with high affinity to the leukemia-inhibitory factor (LIF)/OSM receptor (1). This cytokine, along with other members of a cytokine superfamily, including IL-6, ciliary neurotrophic factor (CNTF), LIF, and IL-11, has a gp130 receptor component involved in signal transduction across the cell membrane (2–4). These cytokines first bind with high affinity to their specific receptor α chain. The individual cytokine receptor complexes then each associate with the same β signal–transducing transmembrane glycoprotein, gp130. gp130 is recruited either as a homodimer or in association with the LIF receptor for signal transduction pathways induced by IL-6, LIF, and CNTF (5–7). In the case of OSM, the molecular structure of its specific receptor has not yet been described; however, OSM has been shown to bind directly to gp130 (8, 9).

Two membrane-proximal regions in gp130 have been proposed to be important for signal transduction (10). These domains are critical for receptor association with members of the Janus family of protein tyrosine kinases (PTKs), such as JAK1 and JAK2 (11–13). Previous studies have demonstrated the involvement of tyrosine and serine/threonine kinases in OSM-mediated intracellular signaling pathways leading to activation of immediate early response genes (14). For example, studies in TF-1 cells have shown that OSM induces tyrosine phosphorylation of JAK2 and that this kinase physically associates with gp130 (15). Moreover, OSM and IL-6 have been shown to stimulate tyrosine phosphorylation and mitogen-activated protein (MAP) kinase (16, 17). MAP kinase activation by receptor-associated PTKs proceeds by way of a cascade that is dependent on Grb2, the guanine nucleotide exchange protein son of sevenless (Sos) and Ras (18, 19). Grb2 is a key molecule in this association through the ability of its SH2 domain to bind to receptor or receptor-associated, tyrosine-phosphorylated proteins. Sos binds to the SH3 domain of Grb2 and is involved in exchange of GDP and GTP in the activation of Ras (20, 21).

The present study demonstrates that treatment with OSM causes tyrosine phosphorylation of JAK2, which then binds directly to the SH2 domain of Grb2. The results further indicate that OSM-induced signaling in U266B1 cells involves formation of a JAK2–Grb2–Sos complex.

Materials and Methods

Cell Culture. U226B1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine se-

[1] *Abbreviations used in this paper:* CNTF, ciliary neurotrophic factor; DTT, dithiothreitol; LIF, leukemia inhibitory factor; OSM, oncostatin M; PTK, protein tyrosine kinase.

rum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY). Cells were treated with 100 ng/ml of rOSM (Bristol Meyers Squibb Pharmaceuticals Research Institute, Seattle, WA).

Reagents and Antibodies. Anti-Grb2 and anti-Sos antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA); anti-JAK2, anti-JAK1, and anti-Tyk2 polyclonal antibodies were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The glutathioinine *S*-transferase (GST)Grb2 (full-length), GST-Grb2 SH2, GST-Grb2 N-SH3, and GST-Grb2 C-SH3 fusion proteins were purchased from Santa Cruz Biotechnology. Antiphosphotyrosine (anti–P-Tyr, 4G10) antibody was provided by Dr. Thomas Roberts (Dana-Farber Cancer Institute, Boston, MA).

Immunoprecipitation and Immunoblotting. Cell lysates were prepared by resuspending U266B1 cells for 30 min on ice in lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM PMSF, 1 mM sodium vanadate, 1 mM dithiothreitol (DTT), 10 mM sodium fluoride, 1% Brij-96, and 10 μg/ml each of leupeptin and aprotinin. Equal amounts of proteins (250–300 μg) were subjected to immunoprecipitation with the indicated antibodies, and immune complexes were precipitated with protein A-Sepharose (Pharmacia Biotechnology, Inc., Piscataway, NJ). The resulting precipitates were washed four times with lysis buffer and resolved by SDS-PAGE under reducing conditions. Proteins were then transferred to nitrocellulose filters, blocked by incubation in 5% dry milk in PBST (0.05% Tween-20 in PBS), and probed with the indicated antibodies. Blots were then developed by enhanced chemiluminesence (ECL; Amersham Corp., Arlington Heights, IL). Nitrocellulose blots were stripped by incubation in 62.5 mM Tris-HCL, pH 6.8, containing 100 mM β-ME and 2% SDS, for 30 min at 50°C. The blots were further washed twice in PBST, reblocked with 5% dry milk in PBST, and reprobed with another antibody.

Fusion Protein Binding Assays, Peptide Competition Assays, and Far Western Analysis. The fusion proteins GST, GST-Grb2 (full-length), GST-Grb2 SH2, GST-Grb2 N-SH3, or GST-Grb2 C-SH3 were purified by affinity chromatography using glutathione–Sepharose beads and equilibrated in lysis buffer. Cell lysates were incubated with 2 μg of immobilized GST, GST-Grb2, GST-Grb2 SH2, GST-Grb2 N-SH3, or GST-Grb2 C-SH3 fusion proteins for 2 h at 4°C. The resulting protein complexes were washed three times with lysis buffer containing 0.1% detergent and boiled for 5 min in SDS buffer. The complexes were then separated by 7.5% SDS-PAGE and subjected to silver staining or immunoblot analysis with anti-JAK2 kinase antibody.

Peptides were synthesized using Fmoc-Tyr (PO_3Me_2)-OH for incorporation of phosphotyrosine and were subsequently purified by ether precipitation and preparative reverse-phase HPLC. Amino acid analysis was used to confirm the sequence of JAK2-derived phosphopeptide. The fusion proteins were first incubated with or without 50 μM tyrosine-phosphorylated peptide [IT-PV_pYHNMFALM], followed by incubation with the lysates from OSM-treated U266B1 cells; adsorbates were analyzed by immunoblotting with anti-JAK2.

For Far Western analysis, the lysates from OSM-treated cells were subjected to immunoprecipitation with anti-JAK2 or preimmune rabbit serum. The proteins were resolved by 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The filters were first incubated with purified GST-Grb2 fusion protein or with purified GST alone (negative control) at room temperature for 2 h and then analyzed by immunoblotting with anti-Grb2.

JAK2 Autophosphorylation, Reprecipitation, and Binding Assays. Cell lysates were immunoprecipitated with anit-JAK2 antibody. After washing three times with lysis buffer containing 0.1% Brij-96 and once with kinase buffer (20 mM Hepes, pH 7.6, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT), in vitro kinase assays were performed by adding 10 μCi [γ-^{32}P]ATP for 15 min at 30°C. The proteins were analyzed by 7.5% SDS-PAGE and autoradiography. JAK2 protein was excised from the gel and eluted at 37°C in elution buffer (50 mM NH_4HCO_3, 1% SDS, and 1 mM DTT). The eluate was diluted 10-fold with lysis buffer and subjected to reprecipitation with anti-JAK2, GST, GST-Grb2 (full length), or GST-Grb2 SH2. The precipitates were analyzed by 7.5% SDS-PAGE and autoradiography.

Results and Discussion

Previous studies have demonstrated that OSM induces proliferation of human plasmacytoma (22) and myeloma cells (23). Other studies in 3T3-L1 preadipocytes have demonstrated that OSM can induce tyrosine phosphorylation of several proteins (15–17, 24). JAK-family tyrosine kinases have been shown to be involved in the signaling pathways triggered by various cytokines, including erythropoietin, IL-3, growth hormone, prolactin, and interferons (25–29). Moreover, other members of the OSM cytokine family, such as IL-6, have been shown to activate JAK kinases differentially in various cell types (30–32). In the present study, we stimulated U266B1 cells with OSM and analyzed total cell lysates for proteins phosphorylated at tyrosine residues. OSM induced several tyrosine-phosphorylated proteins with molecular weights ranging from 40 to 150 kD (Fig. 1 *A* and data not shown). The molecular mass of one such protein is ~120–130 kD and is thus similar to that of JAK-family tyrosine kinases, including JAK1, JAK2, and Tyk2 (Fig. 1 *A*) (25). To determine whether the 120–130 kD protein is a member of the JAK family, we assayed for reactivity of that protein with anti-JAK1, anti-JAK2, and anti-Tyk2 antibodies. In contrast with JAK1 and Tyk2, immunoblot analysis with anti-JAK2 demonstrated reactivity with 130-kD protein (Fig. 1 *A* and data not shown). Taken together, these findings suggest that tyrosine phosphorylation of JAK2 is constitutively low in U266B1 cells and that treatment with OSM increases it three- to fourfold.

To examine whether OSM induces activation of JAK2 kinase in these cells, we treated U266B1 cells with OSM for various time intervals, and performed in vitro immune complex kinase assays on the anti-JAK2 immunoprecipitates. Activation of JAK2 was detectable 2 min after OSM stimulation and returned to near baseline levels at 30 min (Fig. 1 *B*). OSM at 30 ng/ml was sufficient to induce activation of JAK2 kinase (Fig. 1 *C*).

Although OSM has been shown to be cross-linked to a 150–160-kD membrane protein (33), the specific composition and the basis for activation of the OSM receptor are presently unclear. Studies of TF-1 cells have shown that OSM induces tyrosine phosphorylation of JAK2 and that this kinase physically associates with gp130 (4, 15). Furthermore, recent studies have demonstrated that OSM or its related cytokines initiate signaling cascades involving di-

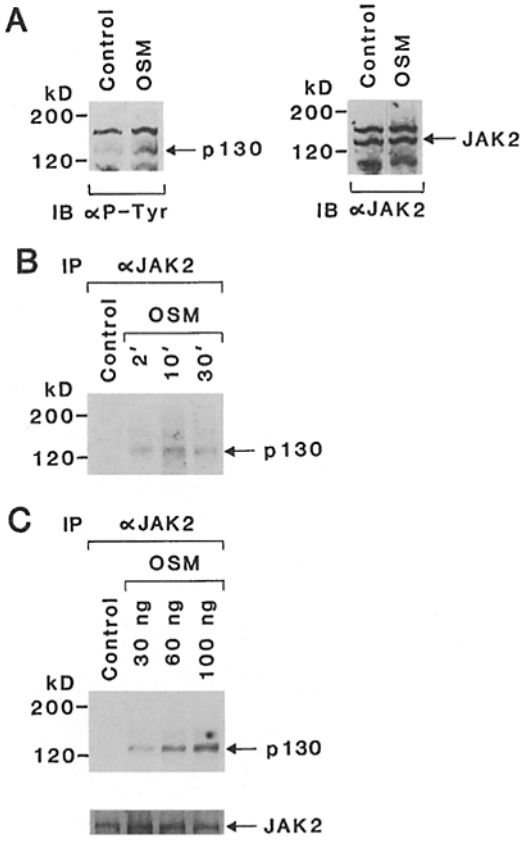

Figure 1. OSM induces tyrosine phosphorylation and activation of JAK2 kinase. (*A*) U266B1 cells were stimulated with 100 ng/ml OSM for 10 min at 37°C and then lysed in 1% Brig-96 lysis buffer. Equal amounts of proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting with anti-P-Tyr or anti-JAK2. Reactivity was determined by ECL. (*B* and *C*) U266B1 cells were treated with either 100 ng/ml OSM for the indicated times (*B*) or at the indicated concentrations of OSM for 10 min (*C*). Total cell lysates were prepared and subjected to immunoprecipitation with anti-JAK2. In vitro immune complex kinase assays were performed by providing [γ-^{32}P]ATP. The proteins were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose filters, and exposed. Filters were stripped and reimmunoblotted with anti-JAK2 (*C, bottom*).

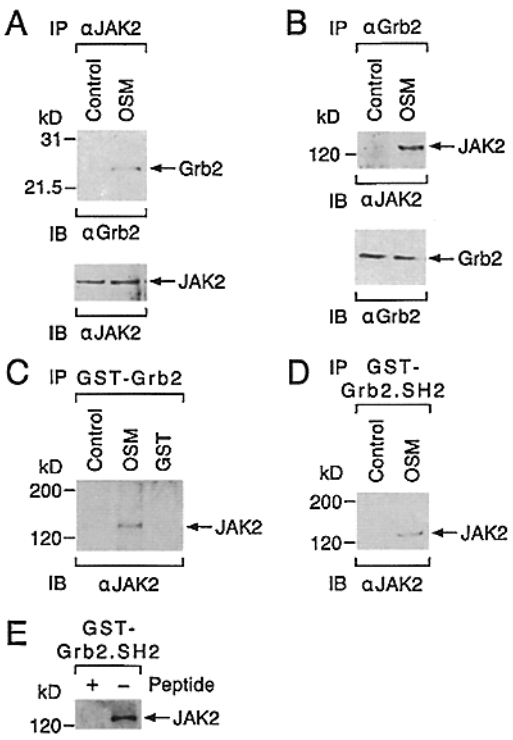

Figure 2. JAK2 associates with Grb2. (*A*) U266B1 cells were stimulated with 100 ng/ml OSM for 10 min. Total cell lysates were immunoprecipitated with anti-JAK2, and the immune complexes were then subjected to immunoblotting with anti-Grb2. Reprobing of the blots with anti-JAK2 demonstrated equal amounts of protein. (*B*) Anti-Grb2 immunoprecipitates were immunoblotted with anti-JAK2, and blots were subsequently reprobed with anti-Grb2 to show equal amounts of protein. (*C*) Lysate from OSM-treated cells was incubated with GST immobilized on glutathione–Sepharose, and lysates from control or OSM-treated U266B1 cells were incubated with immobilized GST-Grb2 (full-length) fusion protein. The adsorbed proteins were separated by 7.5% SDS-PAGE and subjected to immunoblotting with anti-JAK2. (*D*) Lysates from control and OSM-treated U266B1 cells were incubated with GST-Grb2 SH2. The adsorbates were analyzed by immunoblotting with anti-JAK2. (*E*) GST-Grb2 SH-2 fusion protein was incubated (1 h, 4°C) in the presence (+) or absence (−) of 50 μM of tyrosine-phosphorylated synthetic peptide [ITPV$_P$YHNMFALM] followed by incubation with lysates from OSM-treated U266 cells; adsorbates were analyzed by immunoblotting with anti-JAK2.

verse proteins, such as phospholipase C–γ, phosphatidylinositol 3–kinase (PI-3K), Shc, Grb2, Raf-1, and ERK-1/2 (34). Because these studies suggested the involvement of JAK2 in OSM-induced signaling, anti-JAK2 immunoprecipitates were analyzed for potential binding proteins in U266B1 cells. The finding by Coomassie blue staining that a 26-kD protein coprecipitates with JAK2 kinase raised the possibility of an interaction with the SH2/SH3 domain–containing adaptor protein Grb2. Indeed, immunoblot analysis of anti-JAK2 immunoprecipitates with anti-Grb2 revealed increased reactivity with a 26-kD protein after OSM treatment (Fig. 2 *A*). To provide additional support for an association between Grb2 and JAK2 kinase, anti-Grb2 immunoprecipitates were analyzed for reactivity with anti-JAK2. The results demonstrate that Grb2 associates with JAK2 and that this association is increased by OSM treatment (Fig. 2 *B*). In these experiments, reprobing the blots with either anti-JAK2 or anti-Grb2, respectively, demonstrated equal amounts of proteins in each lane (Fig. 2 *A* and *B, lower panel*). The interaction between Grb2 and JAK2 kinase was further examined by incubating lysates from control and OSM-treated U266B1 cells with a GST-Grb2 (full-length) fusion protein. Analysis of the adsorbates by immunoblotting with anti-JAK2 revealed increased reactivity with a 130-kD protein in the OSM-treated cells (Fig. 2 *C*).

To define the domains of Grb2 involved in the association with JAK2, lysates from control and OSM-treated U266B1 cells were incubated with GST fusion proteins prepared from the SH3 (COOH- and NH_2-terminal) and SH2 domains of Grb2. Adsorbates obtained with the GST-Grb2 C-SH3 and GST-Grb2 N-SH3 fusion proteins exhibited little, if any, reactivity with anti-JAK2 (data not

shown). In contrast, adsorbates from GST-Grb2 SH2 revealed increased binding of JAK2 in the OSM-stimulated cells (Fig. 2 *D*). Reprobing the same blot with anti–P-Tyr revealed increases in tyrosine phosphorylation of JAK2 (data not shown). To define further the site in JAK2 responsible for association with Grb2, we identified one potential sequence [ITPV$_P$YHNMFALM] in the NH_2-terminal domain of JAK2 and used tyrosine-phosphorylated synthetic peptide in competition assays. Preincubation of GST-Grb2 SH2 fusion protein with this peptide inhibited binding of JAK2 by lysates of OSM-treated U266 cells (Fig. 2 *E*), whereas no detectable inhibition of JAK2 binding was observed in control lysates without competitor peptide (Fig. 2 *E*). These findings confirmed that the OSM-induced association between JAK2 and Grb2 is mediated primarily through the SH2 domain of Grb2 and that this association is tyrosine phosphorylation dependent.

To determine whether the interaction between Grb2 and JAK2 is direct, we subjected JAK2 immunoprecipitates from OSM-treated U266B1 cells to autophosphorylation. The ^{32}P-labeled JAK2 (Fig. 3 *A*, lane *1*) was excised (Fig. 3 *A*, lane *2*) and eluted from SDS-PAGE gels and then incubated with GST, GST-Grb2 SH2, or GST-Grb2 (full length) (Fig. 3 *A*, lanes *3–5*). One portion of the labeled JAK2 protein was also subjected to immunoprecipitation, with anti-JAK2 as a positive control (lane *6*). Analysis of the second precipitates demonstrated direct binding of the SH2 domain of Grb2 to JAK2 (Fig. 3 *A*). To examine further the direct Grb2–JAK2 interaction, anti-JAK2 immunoprecipitates from OSM-treated cell lysates were resolved on SDS-PAGE and transferred to nitrocellulose. Filters were then incubated either with purified GST-Grb2 fusion protein or with GST (as a negative control) and the blots analyzed by immunoblotting with anti-Grb2. The appearance of p130-kD protein in the filter incubated with GST-Grb2 confirmed direct association between JAK2 and Grb2 proteins (Fig. 3 *B*).

Grb2 adaptor protein has been shown to link receptors or receptor-associated PTKs to the guanine nucleotide exchange protein Sos and thus to the Ras activation pathway (35–37). To study the potential of JAK2 kinase in the Grb2–Sos complex, we analyzed anti-JAK2 immunoprecipitates by immunoblotting with anti-Sos. While little binding of Sos was observed in the anti-JAK2 immunoprecipitates in control cells, this association was increased in OSM-stimulated lysates (Fig. 3 *C*, *left*). In the converse experiment, anti-Sos immunoprecipitates were assayed for reactivity with JAK2. The results demonstrate an increased reactivity with JAK2 in OSM-stimulated U266B1 cells (Fig. 3 *C*, *right*). Taken together, these findings indicate that the JAK2–Sos interaction is mediated through Grb2.

It is not clear how binding of Grb2 to JAK potentiates the ability of Grb2 to activate Sos. The interaction of Grb2 with JAK2 may serve to translocate Sos adjacent to Ras, which is located in the plasma membrane. Another possibility is that the binding of Grb2 or JAK2 promotes the tyrosine phosphorylation of Sos by JAK2, thus stimulating guanine nucleotide–releasing activity. The studies reported

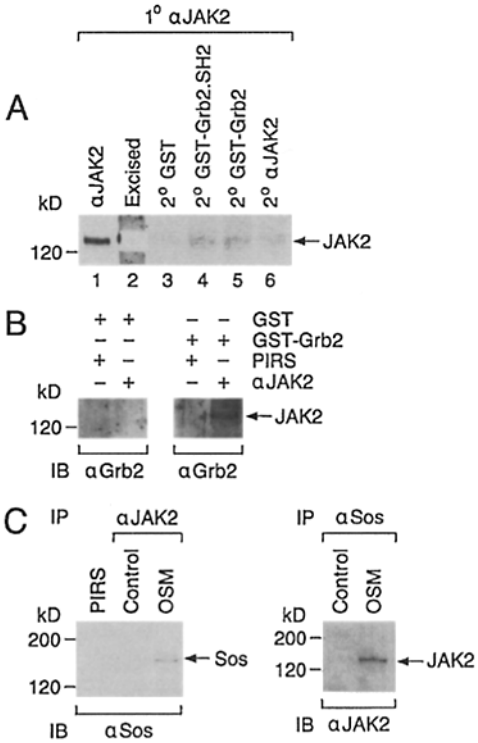

Figure 3. Demonstration of direct association of JAK2 with Grb2 SH2 domain. (*A*) OSM-treated cell lysates were immunoprecipitated with anti-JAK2 antibodies (1°). After in vitro autophosphorylation, the protein was separated by 7.5% SDS-PAGE and analyzed by autoradiography (lane *1*). The JAK2 protein was excised from the gel (lane *2*) and eluted at 4°C in elution buffer. The eluate was diluted 10-fold with lysis buffer and subjected to reprecipitation (2°) with indicated GST fusion proteins (lanes *3–5*) and anti-JAK2 (lane *6*). The proteins were resolved by 7.5% SDS-PAGE and analyzed by autoradiography. (*B*) The lysates from OSM-treated cells were subjected to immunoprecipitation with anti-JAK2 and preimmune rabbit serum. The proteins were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane. The filters were first incubated with purified GST-Grb2 fusion protein or with purified GST fusion protein alone (negative control) and then immunoblotted with anti-Grb2. (*C*) Control and OSM-treated cell lysates were subjected to immunoprecipitation with anti-JAK2 or preimmune rabbit serum (negative control). The immunoprecipitates were analyzed by immunoblotting with anti-Sos (*left*). Anti-Sos immunoprecipitates were also analyzed by immunoblotting with anti-JAK2 (*right*).

here demonstrate that, as shown for other cytokines, OSM stimulation of growth pathways leading to Ras activation is mediated by Grb2. However, in contrast to growth factor receptors that interact directly with the Grb2–Sos complex, stimulation by OSM leads to phosphorylation of JAK2 kinase. The interaction of Grb2–Sos complex with JAK2 in OSM-stimulated myeloma cells may provide an additional level of control, conferred by the OSM receptor, of this pivotal signaling pathway. In summary, our results demonstrate that OSM treatment of U266B1 human myeloma cells induces tyrosine phosphorylation and activation of JAK2 kinase and potentiates its association with the SH2 domain of Grb2. Since Sos was also detected in the complexes of JAK2–Grb2, we conclude that the JAK2–Grb2–Sos complexes are involved in OSM-induced growth of U266B1 cells.

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