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# JAK2 Associates with the $\beta_c$ Chain of the Receptor for Granulocyte-Macrophage Colony-Stimulating Factor, and Its Activation Requires the Membrane-Proximal Region

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**The high-affinity receptor for granulocyte-macrophage colony-stimulating factor (GM-CSF) consists of a unique α chain and a $\beta_c$ subunit that is shared with the receptors for interleukin-3 (IL-3) and IL-5. Two regions of the $\beta_c$ chain have been defined; these include a membrane-proximal region of the cytoplasmic domain that is required for mitogenesis and a membrane-distal region that is required for activation of Ras, Raf-1, mitogen-activated protein kinase, and S6 kinase. Recent studies have implicated the cytoplasmic protein tyrosine kinase JAK2 in signalling through a number of the cytokine receptors, including the IL-3 and erythropoietin receptors. In the studies described here, we demonstrate that GM-CSF stimulation of cells induces the tyrosine phosphorylation of JAK2 and activates its in vitro kinase activity. Mutational analysis of the $\beta_c$ chain demonstrates that only the membrane-proximal 62 amino acids of the cytosolic domain are required for JAK2 activation. Thus, JAK2 activation is correlated with induction of mitogenesis but does not, alone, activate the Ras pathway. Carboxyl truncations of the α chain, which inactivate the receptor for mitogenesis, are unable to mediate GM-CSF-induced JAK2 activation. Using baculovirus-expressed proteins, we further demonstrate that JAK2 physically associates with the $\beta_c$ chain but not with the α chain. Together, the results further support the hypothesis that the JAK family of kinases are critical to coupling cytokine binding to tyrosine phosphorylation and ultimately mitogenesis.**

Hematopoiesis is regulated through the interaction of one or more of a variety of cytokines with their cognate receptors. Granulocyte-macrophage colony-stimulating factor (GM-CSF) regulates the proliferation and differentiation of cells at various stages of differentiation along the myeloid lineages (1, 17). The functional, high-affinity receptor for GM-CSF consists of two subunits, each of which is a member of the cytokine receptor superfamily (19, 20). The binding of GM-CSF occurs through an α subunit of 60 to 80 kDa, which alone binds GM-CSF with low affinity. Association of the α chain with a 140-kDa $\beta_c$ chain results in the formation of a high-affinity binding site for GM-CSF. The $\beta_c$ chain also associates with α subunits that specifically bind interleukin-3 (IL-3) and IL-5 and similarly contributes to the formation of high-affinity binding sites for these ligands.

Stimulation of growth factor-dependent cell lines with GM-CSF has been shown to induce a variety of immediate cellular responses, including the rapid tyrosine phosphorylation of the $\beta_c$ subunit and a number of cellular substrates (7, 16, 30); induction of transcription of several immediate-early genes (5); and activation of components of the Ras signalling pathway, including SHC phosphorylation, increases in GTP-bound Ras, activation of Raf-1 kinase, and activation of mitogen-activated protein (MAP) kinase (4, 8, 23, 26, 27). Recent studies (26) have shown that at least two distinct cytoplasmic regions of the $\beta_c$ subunit are required for these functions. A membrane-proximal region of approximately 60 amino acids is essential for induction of c-*myc* and *pim-1* as well as for inducing mitogenesis, while a distal region is required for activation of Ras, Raf-1, MAP kinase, and S6 kinase.

Although considerable evidence demonstrates that induction of protein tyrosine phosphorylation is critical to cytokine function, only recently have the JAK family of kinases been implicated in coupling ligand binding to tyrosine phosphorylation. The JAK family consists of JAK1 (15), JAK2 (29), and TYK2 (9) and is characterized by proteins of approximately 130 kDa that contain a carboxyl kinase domain and a second kinase-like domain and lack SH2 or SH3 domains. JAK2 has been shown to be activated in the responses to erythropoietin (Epo) (36), IL-3 (29), growth hormone (2), G-CSF (35a), and prolactin (3). In contrast, IL-6, ciliary neurotrophic factor (CNTF), and leukemia-inhibitory factor activate JAK1, JAK2, and to some extent TYK2 (22, 31). The JAK kinases have also been shown to play an essential role in interferon signalling. In particular, the response to alpha/beta interferon requires both JAK1 (21) and TYK2 (34), while the response to gamma interferon requires JAK1 (21) and JAK2 (35).

In a number of cases, JAK family members have been shown to associate with the receptors either prior to or following ligand binding. In particular, JAK2 binds to the membrane-proximal region of the Epo receptor (EpoR) (36), a region that has been shown to be required for mitogenesis (18). Similarly, JAK1 and JAK2 associate with the membrane-proximal region of gp130 (22, 31), which is required for mitogenesis. In the studies presented here, we have examined the role of the JAK family members in signalling through the receptor for GM-CSF. The studies demonstrate that JAK2 is activated by GM-CSF binding. Moreover, activation requires a membrane-proximal domain of the $\beta_c$ chain as well as the cytoplasmic

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domain of the α chain. Lastly, JAK2 associates with the $\beta_c$ chain but not the α chain.

## MATERIALS AND METHODS

**Cell lines.** Human TF-1 cells (American Type Culture Collection) were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 10 ng of human IL-3 per ml. BaF3 cells expressing human GM-CSF receptor, either wild type or mutant, have been described elsewhere (25, 26) and were maintained in RPMI 1640 supplemented with 10% FCS and an appropriate cytokine. BaF3/$\alpha\beta_c$, BaF3/$\alpha\beta_{763}$, BaF3/$\alpha\beta_{626}$, and BaF3/$\alpha\beta_{517}$ cells were maintained in either 25 U of murine IL-3 per ml or 20 ng of human GM-CSF per ml. BaF3/$\alpha\beta_{455}$ and BaF3/$\alpha_{328}\beta_c$ cells were maintained in 25 U of murine IL-3 per ml. SF9 cells were maintained in Grace's insect medium supplemented with 10% FCS.

**Reagents.** The antiserum against JAK2 has been described elsewhere (29, 36). Monoclonal antibodies against $p93^{c\text{-}fes}$ (clone F-113) were purchased from Oncogene Science. Rabbit monoclonal antibodies against rat immunoglobulin M were purchased from Calbiochem. Polyclonal antisera against amino acids 23 to 38 of the GM-CSF receptor α chain were prepared by immunizing rabbits with peptide coupled to keyhole limpet hemocyanin followed by affinity purification on a bovine serum albumin (BSA)-peptide coupled solid support (13). Polyclonal antisera against the extracellular domain of the GM-CSF receptor β chain were prepared by immunizing rabbits with a glutathione *S*-transferase—β-chain fusion protein followed by affinity purification using the fusion protein cross-linked to glutathione-agarose beads. The monoclonal antiphosphotyrosine antibody 1G2 (Oncogene Science) was used for immunoprecipitations. The monoclonal antiphosphotyrosine antibodies PY20 (ICN) and 4G10 (a gift from Brian Druker, University of Oregon) were used for Western blotting (immunoblotting).

**Immunoprecipitation, SDS-PAGE, and Western blotting.** Prior to stimulation, cells were starved for 14 to 16 h in RPMI 1640 supplemented with 1% FCS. Cells were treated with growth factor and were subsequently lysed in lysis buffer (1% Triton X-100, 50 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 0.1 mM $Na_3VO_4$, 5 mM EDTA, 0.1% BSA, 0.05 mg of phenylmethylsulfonyl fluoride per ml, 10 mM Tris [pH 7.6]). Lysates were cleared of debris at 12,000 × *g* for 10 min, and the supernatants were incubated in the presence of the designated sera or monoclonal antibody for 2 h. Immune complexes were precipitated with protein A-Sepharose (Sigma) and extensively washed in lysis buffer without BSA, and proteins then were eluted with sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Eluted proteins were separated on 7% gels and transferred to nitrocellulose. Filters were probed with designated sera or antibodies and visualized with the ECL (enhanced chemiluminescence) detection system (Amersham) as directed by the manufacturer.

**Kinase assays.** Immunoprecipitated proteins on protein A-Sepharose were washed with kinase buffer (50 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mM $Na_3VO_4$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.4]) and subsequently were incubated for 30 min at room temperature with an equal volume of kinase buffer containing 0.25 mCi of [$\gamma$-$^{32}$P]ATP (New England Nuclear) per ml. After extensive washing, proteins were eluted with sample buffer for SDS-PAGE and separated on 7% gels. $^{32}$P-containing proteins were visualized by autoradiography.

**Baculovirus expression.** Baculovirus containing a human EpoR (6), human GM-CSF receptor α chain (10), human $\beta_c$ chain (14), or murine Jak2 (29) cDNA were prepared by cloning the appropriate cDNAs into the transfer vector pVL1392. Plasmid constructs then were cotransfected with linearized Baculogold baculovirus DNA (Pharmingen) into *Spodoptera frugiperda* SF9 or SF21 cells as previously described (33) and as recommended by the manufacturer. Expression of proteins in SF9 or SF21 cells was achieved by incubating cells in the presence of one or more high-titer recombinant viruses (multiplicity of infection of 5 to 10). At 48 to 72 h after infection, cells were lysed and analyzed by immunoprecipitation and Western blotting.

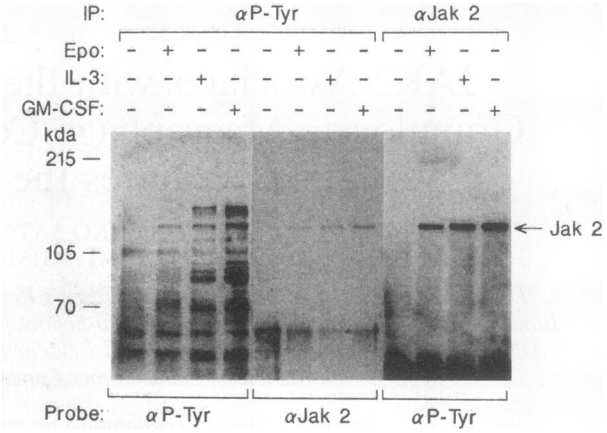


FIG. 1. GM-CSF, Epo, and IL-3-induced tyrosine phosphorylation in TF-1 cells. TF-1 cells were stimulated with no factor, Epo (20 U/ml), IL-3 (200 ng/ml), or GM-CSF (600 ng/ml) for 10 min. Cell lysates were prepared and immunoprecipitated (IP) with an antiphosphotyrosine monoclonal antibody (αP-Tyr) or a JAK2 antiserum (αJak 2). Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to phosphotyrosine or JAK2. Each lane represents precipitated proteins from $10^7$ TF-1 cells.

## RESULTS

**GM-CSF-induced phosphorylation and activation of JAK2.** We have previously demonstrated the tyrosine phosphorylation and activation of JAK2 in the responses to Epo (36) and IL-3 (29). The utilization of a common subunit in the GM-CSF and IL-3 receptors and the general similarity of induced tyrosine phosphorylation suggested the possibility that GM-CSF would also phosphorylate and activate JAK2. To explore the potential roles of JAK2 in the response to GM-CSF, TF-1 cells were stimulated with GM-CSF, and the effect on the tyrosine phosphorylation of JAK2 was examined (Fig. 1). For comparison, the responses to IL-3 and Epo are also shown. Immunoprecipitation of phosphotyrosine-containing proteins followed by Western blotting with an antiphosphotyrosine monoclonal antibody demonstrated that all three growth factors induced protein tyrosine phosphorylation of similar substrates. Probing of antiphosphotyrosine immunoprecipitates with an antiserum against JAK2 demonstrated that JAK2 was present in immunoprecipitates from cells stimulated with Epo, IL-3, or GM-CSF but not from unstimulated cells. The growth factor-induced tyrosine phosphorylation of JAK2 was also examined by immunoprecipitation of the kinases and probing the blots with a monoclonal antibody against phosphotyrosine. As illustrated in Fig. 1, Epo, IL-3, and GM-CSF induced the tyrosine phosphorylation of JAK2. Therefore, GM-CSF, like IL-3 or Epo, results in the tyrosine phosphorylation of JAK2.

Epo- and IL-3-induced JAK2 tyrosine phosphorylation oc-

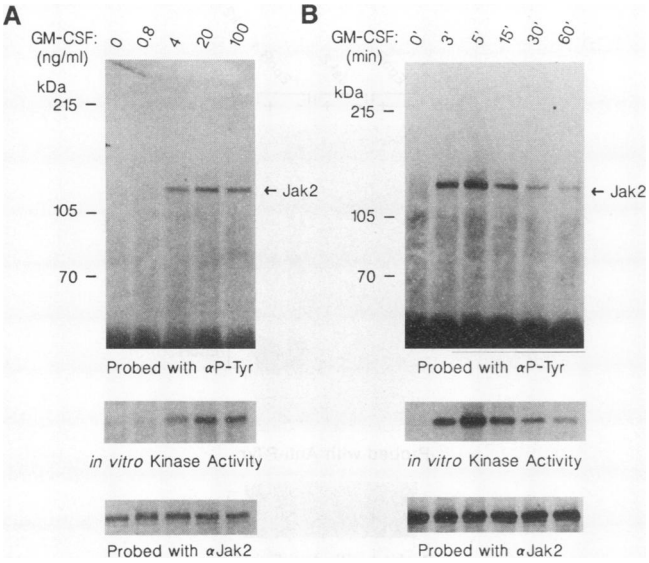


FIG. 2. Dose- and time-dependent phosphorylation and activation of JAK2. TF-1 cells were stimulated with increasing concentrations of GM-CSF for 10 min (A) or with 10 ng of GM-CSF per ml for increasing intervals of time (B). Following stimulation, JAK2 was isolated by immunoprecipitation and analyzed by Western blotting with an antiphosphotyrosine monoclonal antibody (αP-Tyr) or an anti-JAK2 antiserum (αJak2). A portion of the immunoprecipitated JAK2 was also subjected to an in vitro kinase assay.

curs in a dose- and time-dependent fashion and is correlated with the activation of JAK2 in vitro tyrosine kinase activity (29, 36). The kinetics and dose-response properties of the response of TF-1 cells to GM-CSF are shown in Fig. 2. Tyrosine phosphorylation of JAK2 occurred rapidly following GM-CSF stimulation and occurred at concentrations of GM-CSF that are sufficient to induce mitogenesis. The in vitro kinase activity of JAK2 immunoprecipitates was also examined (Fig. 2). Similar to the induction of tyrosine phosphorylation, GM-CSF induced the activation of kinase activity in a dose-dependent manner and with comparable kinetics.

**The membrane-proximal cytoplasmic region is required for JAK2 activation.** Previous studies (26) have defined two cytoplasmic domains of the GM-CSF receptor $\beta_c$ chain. Carboxyl truncation at amino acid 763 does not affect the ability of the receptor to induce either proliferation or a series of cellular responses associated with activation of the Ras pathway (Fig. 3). However, a carboxyl truncation at amino acid 626 results in the loss of the ability to activate the Ras pathway, including SHC phosphorylation, increased GTP-bound Ras, and activation of Raf-1, MAP kinase, and S6 kinase, while not affecting mitogenesis. Importantly, the truncated receptors that retained mitogenic activity were still sensitive to herbimycin A, implicating a protein tyrosine kinase in their activity. Further truncation of the $\beta_c$ chain to amino acid 455 results in the loss of mitogenesis. It was therefore important to determine which domain might be required for JAK2 tyrosine phosphorylation and/or activation of JAK2 in vitro kinase activity. The results obtained with the various mutants expressed in BaF3 cells are shown in Fig. 4. BaF3 cells expressing the wild-type GM-CSF receptor α and $\beta_c$, $\beta_{763}$, $\beta_{626}$, or $\beta_{517}$ each were able to phosphorylate and activate JAK2 in response to GM-CSF. However, the extent of JAK2 phosphorylation seen in cells expressing the $\beta_{517}$ mutant was consistently reduced, consistent with the weak mitogenicity seen with cells expressing this mutant (25, 26). In contrast, no phosphorylation or activation of JAK2 was detected in cells expressing the most truncated receptor, $\beta_{455}$. Thus, the membrane-proximal region between amino acids 455 and 517 of the $\beta_c$ chain is required both for JAK2 phosphorylation and activation and for mitogenesis.

Truncations of the GM-CSF receptor α-chain cytoplasmic domain have also been shown to inactivate the receptor for mitogenesis (24, 25). As summarized in Fig. 3, BaF3 cells expressing a truncated α chain, $\alpha_{328}$, and a wild-type $\beta_c$ are incapable of proliferating in response to GM-CSF, nor is JAK2 phosphorylated or activated following GM-CSF treatment (Fig. 4). Thus, the cytoplasmic domains of both the α and $\beta_c$ chains are required for JAK2 activation, and among the mutants examined, there is a complete correlation between the ability to activate JAK2 and to support mitogenesis.

**Association between JAK2 and EpoR or the β chain of the GM-CSF receptor in insect cells.** We have previously demonstrated that JAK2 associates in vitro with glutathione *S*-

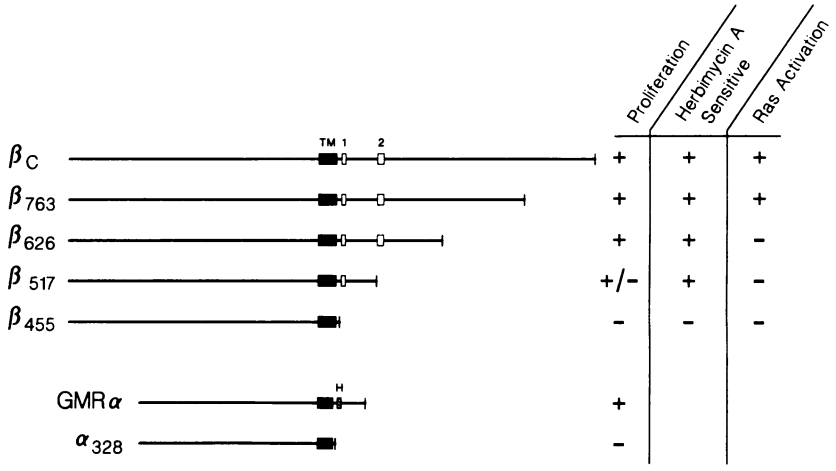


FIG. 3. GM-CSF receptor mutants. Wild-type and carboxyl truncation mutants of human $\beta_c$ and GM-CSF receptor α are diagrammed. Locations of transmembrane domains (TM; filled box) and the box 1/box 2 homology regions (open boxes) are shown for the $\beta_c$ chain. A region of homology shared by the α subunits of receptors for GM-CSF, IL-3, and IL-5 is also shown (H; hatched box). The ability of GM-CSF to regulate proliferation or Ras activation in BaF3 cells expressing the GM-CSF receptor α chain (GMRα) and $\beta_c$ or $\beta_c$ mutants is indicated at the right.

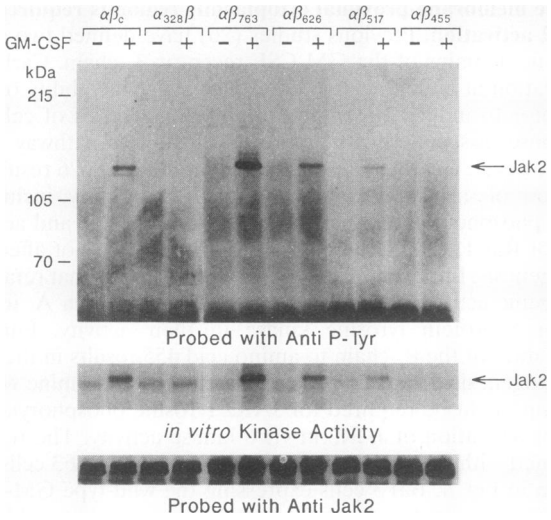


FIG. 4. Activation of JAK2 by GM-CSF receptor mutants. BaF3 cells expressing wild-type or mutated receptors (see Fig. 3) were stimulated with or without GM-CSF (1 μg/ml) for 10 min. Following stimulation, JAK2 was isolated by immunoprecipitation and analyzed by Western blotting with an antiphosphotyrosine monoclonal antibody (αP-Tyr) or an antiserum against JAK2. A portion of the immunoprecipitated JAK2 also was subjected to an in vitro kinase assay.

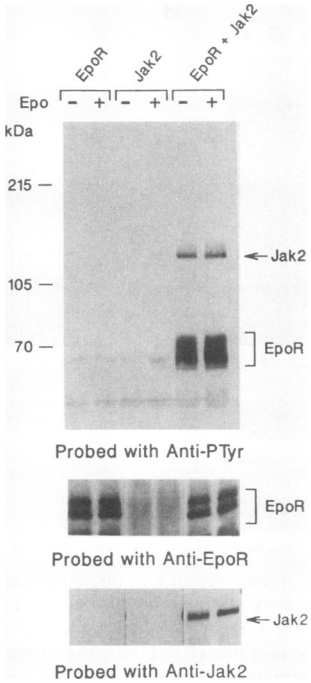


FIG. 5. EpoR associates with JAK2 in insect cells. SF9 cells were infected with a baculovirus expression vector for EpoR or JAK2 or were coinfected with both viruses. At 40 h after infection, SF9 cells were treated with no factor (−) or with 10 U of Epo per ml (+) for 10 min. Cells then were lysed and immunoprecipitated with anti-EpoR sera. Precipitated proteins were analyzed by Western blotting using an antiphosphotyrosine monoclonal antibody (anti-PTyr), anti-EpoR sera, or anti-JAK2 sera.

transferase fusion proteins containing the membrane-proximal domain of EpoR (36). To explore the potential association of JAK2 with the GM-CSF receptor α and β chains, we used baculovirus expression vectors to look for association in insect cells. For comparison, we also used expression vectors containing EpoR. Baculovirus expression of JAK2 in SF9 insect cells alone resulted in the constitutive tyrosine phosphorylation of JAK2 in vivo as well as constitutive activation of JAK2 in vitro kinase activity (data not shown). The results obtained with expression vectors for EpoR and JAK2 are shown in Fig. 5. In these experiments, EpoR was expressed in SF9 cells either alone or in combination with JAK2. Following 2 days of infection, cells were lysed, EpoR was immunoprecipitated, and the immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. The blots were then probed with antibodies against phosphotyrosine, EpoR, or JAK2. As illustrated, infection of cells with the EpoR expression vector resulted in the production of EpoR, but this protein was not detectably tyrosine phosphorylated. Coinfection of cells with viruses expressing EpoR and JAK2 similarly resulted in the production of comparable levels of EpoR; however, this protein was readily detectable with antibodies against phosphotyrosine. Moreover, JAK2 was readily detectable in the EpoR immunoprecipitates either with antibodies against phosphotyrosine or with an antiserum against JAK2. These results demonstrate that EpoR associates with JAK2 and is tyrosine phosphorylated in insect cells. The levels of association and tyrosine phosphorylation were comparable in the presence or absence of ligand.

We next examined whether JAK2 associated with the GM-CSF receptor α and/or β chains. Insect cells were infected with various combinations of viruses, the cells were lysed, the $\beta_c$ chain was immunoprecipitated, the immunoprecipitates were resolved by SDS-PAGE and blotted to nitrocellulose, and the blots were probed with an antiserum against JAK2. As shown in Fig. 6A, JAK2 coimmunoprecipitated with the $\beta_c$ chain in cells infected with expression vectors for JAK2 and $\beta_c$. The additional expression of the α chain had little effect on the amount of JAK2 detected in $\beta_c$ immunoprecipitates, nor did the presence of GM-CSF affect the coimmunoprecipitation in insect cells infected with viruses expressing the α and $\beta_c$ chains (data not shown). In contrast, JAK2 was not detected in $\beta_c$ immunoprecipitates from controls, including uninfected cells, cells infected with only JAK2, or cells infected with JAK2 and the α chain. Similarly, JAK2 was not detected by preimmune serum in cells expressing JAK2, the α chain, and the $\beta_c$ chain. Lastly, JAK2 did not coimmunoprecipitate with the α chain in any of the conditions specified above (data not shown).

The ability of JAK2 to associate with the $\beta_c$ chain was also examined by immunoprecipitating JAK2, resolving the precipitates by SDS-PAGE, transferring the proteins to nitrocellulose, and probing the blots with an antiserum against the $\beta_c$ chain. As shown in Fig. 6B, the $\beta_c$ chain coimmunoprecipitated with JAK2 in cells infected with viruses expressing JAK2 and the $\beta_c$ chain. As above, the inclusion of viruses expressing the α chain had no apparent effect on the results, nor was $\beta_c$ detected in JAK2 immunoprecipitates from cells infected with only JAK2 and/or the α-chain virus (data not shown). These data demonstrate that JAK2 specifically associates with the $\beta_c$ subunit of the receptor for GM-CSF.

## DISCUSSION

Our results demonstrate that JAK2 is involved in the response to GM-CSF, extending the list of cytokines that utilize JAK2 in signalling. The response was specific to JAK2, and in particular, we have not detected any phosphorylation or activation of either JAK1 or TYK2 (data not shown). Among

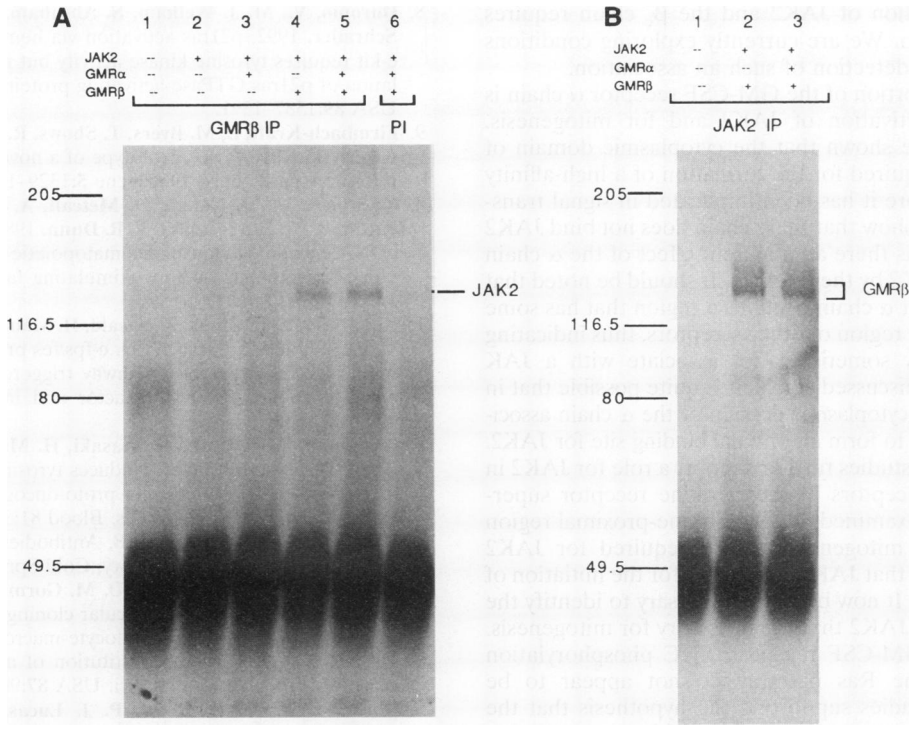


FIG. 6. GM-CSF receptor association with JAK2 in insect cells. SF21 cells were infected with various combinations of baculovirus expression vectors for JAK2 and GM-CSF receptor α or $\beta_c$ chain (GMRα or GMRβ). Following infection, cells were lysed, immunoprecipitated with anti-$\beta_c$ sera (GMRβ IP) or preimmune sera (PI), and subsequently Western blotted with anti-JAK2 sera (A). Alternatively, cell lysates were immunoprecipitated with anti-JAK2 sera (JAK2 IP) and Western blotted with anti-$\beta_c$ sera (B). Sizes are indicated in kilodaltons.

other cytoplasmic protein tyrosine kinases, we have not detected any effects of GM-CSF on the phosphorylation of either Lyn or Tec. We have also been unable to see any effect of GM-CSF on $p93^{c\text{-}fes}$ in TF-1 cells. This is in contrast to recent studies which demonstrated the activation of $p93^{c\text{-}fes}$ in the responses of this cell line to IL-3 and GM-CSF (11) as well as Epo (12). The basis of the differences is not known, since we have used the same cells as well as the same monoclonal antibody against $p93^{c\text{-}fes}$.

Our studies took advantage of a series of well-characterized mutations of the $\beta_c$ chain of the GM-CSF receptor (26). Importantly, previous studies have shown that carboxyl truncation of the $\beta_c$ chain at residue 626 inactivated the receptor for initiation of a series of cellular responses associated with activation of the Ras pathway and induction of c-*fos* and c-*jun*, without affecting mitogenesis or c-*myc* and *pim-1* expression. Our results demonstrate that this mutant is still able to activate JAK2. Thus, JAK2 activation per se is not sufficient for SHC phosphorylation, activation of Ras, activation of Raf-1, or activation of MAP kinase. Activation of this Ras pathway may require another cytoplasmic protein tyrosine kinase which associates with the membrane-distal region of the cytoplasmic domain of the $\beta_c$ chain. Alternatively, the membrane-distal region may be required for binding of initiators of the pathway which are activated by phosphorylation by JAK2.

Previous deletional analysis (25, 26) showed that the distal region of the $\beta_c$ subunit is responsible for activation of the Ras pathway and is dispensable for GM-CSF-dependent proliferation of BaF3 transfectants under normal culture conditions with serum. Although GM-CSF induces short-term cell cycle progression of BaF3 transfectants with $\beta_{626}$ without serum, GM-CSF does not support the long-term proliferation of the cells in the absence of serum. In addition, serum induces activation of the Ras pathway in BaF3 cells. Thus, serum appears to alleviate the requirement for the Ras pathway for GM-CSF-dependent proliferation by the $\beta_c$ deletion mutant lacking the distal region (24a). Irrespective, under serum-free conditions, both the membrane-proximal and membrane-distal regions of $\beta_c$ are required for proliferation.

The activation of JAK2 requires the membrane-proximal region which contains the box 1/box 2 homology domains. Comparable regions of EpoR (36) and gp130 (22, 32) have been shown to be required for activation of JAK family members. On the basis of these studies and in vitro binding experiments, we would propose that the box 1/box 2 site is the primary site of association of JAK family members with cytokine receptors.

The results demonstrate that JAK2 can physically associate with and phosphorylate EpoR as well as the GM-CSF receptor $\beta_c$ chain in insect cells. This association is specific for JAK2 relative to JAK1 or TYK2 (data not shown). The association of JAK2 with the $\beta_c$ chain was not dependent upon, or influenced by, the presence of GM-CSF or the GM-CSF receptor α chain, suggesting that JAK2 and the $\beta_c$ chain may be constitutively associated in cells. However, the high concentrations that are achieved in insect cells often preclude the detection of subtle changes in affinities that would affect associations in mammalian cells. In particular, glutathione *S*-transferase fusion proteins containing the EpoR cytoplasmic domain associate with JAK2 independently of ligand (36), and as demonstrated here, baculovirus-expressed proteins associate independently of ligand. However, recent studies have found that under appropriate conditions of cell lysis, a ligand-dependent association of JAK2 with EpoR can be demonstrated (17a). Therefore, it is

possible that association of JAK2 and the $\beta_c$ chain requires ligand binding in vivo. We are currently exploring conditions that might allow the detection of such an association.

The cytoplasmic portion of the GM-CSF receptor α chain is also required for activation of JAK2 and for mitogenesis. Previous studies have shown that the cytoplasmic domain of the α chain is not required for the formation of a high-affinity receptor, and therefore it has been implicated in signal transduction. Our studies show that the α chain does not bind JAK2 in insect cells, nor was there an apparent effect of the α chain on the binding of JAK2 by the $\beta_c$ chain. It should be noted that the GM-CSF receptor α chain contains a region that has some similarity to the box 1 region of other receptors, thus indicating that this region may sometimes not associate with a JAK kinase. However, as discussed above, it is quite possible that in mammalian cells, the cytoplasmic domain of the α chain associates with the $\beta_c$ chain to form an optimal binding site for JAK2.

In summary, these studies further support a role for JAK2 in signalling through receptors of the cytokine receptor superfamily. In each case examined, the membrane-proximal region that is required for mitogenesis is also required for JAK2 activation, suggesting that JAK2 is essential for the initiation of cellular proliferation. It now becomes necessary to identify the cellular substrates of JAK2 that are necessary for mitogenesis. In the case of the GM-CSF receptor, SHC phosphorylation and activation of the Ras pathway do not appear to be necessary. Recent studies supporting the hypothesis that the 91- and 113-kDa components of the ISGF3 transcription complex are substrates of JAK family members (21, 28, 35) and the identification of 91-kDa related proteins among the proteins that are tyrosine phosphorylated in the response to IL-3 suggest an alternative pathway for regulation of the gene transcription involved in the mitogenic response.

## ACKNOWLEDGMENTS

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## REFERENCES

1. **Arai, K., F. Lee, A. Miyajima, S. Miyatake, N. Arai, and T. Yokota.** 1990. Cytokines: coordinators of immune and inflammatory responses. Annu. Rev. Biochem. **59:**783–836.
2. **Artgetsinger, L. S., G. S. Campbell, X. Yang, B. A. Witthuhn, O. Silvennoinen, J. N. Ihle, and C. Carter-Su.** 1993. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell **74:**237–244.
3. **Campbell, G. S., L. S. Argetsinger, J. N. Ihle, P. A. Kelly, J. A. Rillema, and C. Carter-Su.** Activation of JAK2 tyrosine kinase by prolactin receptors in $Nb_2$ cells and mouse mammary gland explants. Proc. Natl. Acad. Sci. USA, in press.
4. **Carroll, M. P., J. L. Spivak, M. McMahon, N. Weich, U. R. Rapp, and W. S. May.** 1991. Erythropoietin induces raf-1 activation and raf-1 is required for erythropoietin-mediated proliferation. J. Biol. Chem. **266:**14964–14969.
5. **Conscience, J. F., B. Verrier, and G. Martin.** 1986. Interleukin-3-dependent expression of the c-myc and c-fos proto-oncogenes in hemopoietic cell lines. EMBO J. **5:**317–323.
6. **D'Andrea, A. D., H. F. Lodish, and G. G. Wong.** 1989. Expression cloning of the murine erythropoietin receptor. Cell **57:**277–285.
7. **Duronio, V., I. Clark-Lewis, B. Federsppiel, J. S. Wieler, and J. W. Schrader.** 1992. Tyrosine phosphorylation of receptor beta subunits and common substrates in response to interleukin-3 and granulocyte-macrophage colony-stimulating factor. J. Biol. Chem. **267:**21856–21863.
8. **Duronio, V., M. J. Welham, S. Abraham, P. Dryden, and J. W. Schrader.** 1992. p21ras activation via hemopoietin receptors and c-kit requires tyrosine kinase activity but not tyrosine phosphorylation of p21ras GTPase-activating protein. Proc. Natl. Acad. Sci. USA **89:**1587–1591.
9. **Firmbach-Kraft, I., M. Byers, T. Shows, R. Dalla-Favera, and J. J. Krolewski.** 1990. Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. Oncogene **5:**1329–1336.
10. **Gough, N. M., J. Gough, D. Metcalf, A. Kelso, D. Grail, N. A. Nicola, A. W. Burgess, and A. R. Dunn.** 1984. Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. Nature (London) **309:**763–767.
11. **Hanazono, Y., S. Chiba, K. Sasaki, H. Mano, A. Miyajima, K. Arai, Y. Yazaki, and H. Hirai.** 1993. c-fps/fes protein-tyrosine kinase is implicated in a signaling pathway triggered by granulocyte-macrophage colony-stimulating factor and interleukin-3. EMBO J. **12:**1641–1646.
12. **Hanazono, Y., S. Chiba, K. Sasaki, H. Mano, Y. Yazaki, and H. Hirai.** 1993. Erythropoietin induces tyrosine phosphorylation and kinase activity of the c-*fps/fes* proto-oncogene product in human erythropoietin-responsive cells. Blood **81:**3193–3196.
13. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Hayashida, K., T. Kitamura, D. M. Gorman, K. Arai, T. Yokota, and A. Miyajima.** 1990. 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 **87:**9655–9659.
15. **Horak, I. D., R. E. Gress, P. J. Lucas, E. M. Horak, T. A. Waldmann, and J. B. Bolen.** 1991. T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of $p56^{lck}$. Proc. Natl. Acad. Sci. USA **88:**1996–2000.
16. **Isfort, R. J., and J. N. Ihle.** 1990. Multiple hematopoietic growth factors signal through tyrosine phosphorylation. Growth Factors **2:**213–220.
17. **Metcalf, D.** 1989. The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature (London) **339:**27–30.

17a. **Miura, O., N. Nakamura, F. W. Quelle, B. A. Witthuhn, J. N. Ihle, and N. Aoki.** Erythropoietin induces association of the JAK2 protein tyrosine kinase with the erythropoietin receptor in vivo. Blood, in press.

18. **Miura, O., J. L. Cleveland, and J. N. Ihle.** 1993. Inactivation of erythropoietin receptor function by point mutations in a region having homology with other cytokine receptors. Mol. Cell. Biol. **13:**1788–1795.
19. **Miyajima, A., T. Kitamura, N. Harada, T. Yokota, and K. Arai.** 1992. Cytokine receptors and signal transduction. Annu. Rev. Immunol. **10:**295–331.
20. **Miyajima, A., A. L.-F. Mui, T. Ogorochi, and K. Sakamaki.** 1993. Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. Blood **82:**1960–1974.
21. **Muller, M., J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. G. Harpur, G. Barbieri, B. A. Witthuhn, C. Schindler, S. Pellegrini, A. F. Wilks, J. N. Ihle, G. R. Stark, and I. M. Kerr.** 1993. The protein tyrosine kinase JAK1 complements defects in interferon-α/β and -γ signal transduction. Nature (London) **366:**129–135.
22. **Narazaki, M., B. A. Witthuhn, K. Yoshida, O. Silvennoinen, K. Yasukawa, J. N. Ihle, T. Kishimoto, and T. Taga.** 1994. Activation of JAK2 kinase mediated by the IL-6 signal transducer, gp130. Proc. Natl. Acad. Sci. USA **91:**2285–2289.
23. **Okuda, K., J. S. Sanghera, S. L. Pelech, Y. Kanakura, M. Hallek, J. D. Griffin, and B. J. Druker.** 1992. Granulocyte-macrophage colony-stimulating factor, interleukin-3, and steel factor induce rapid tyrosine phosphorylation of p42 and p44 MAP kinase. Blood **79:**2880–2887.
24. **Polotskaya, A., Y. Zhao, M. L. Lilly, and A. S. Kraft.** 1993. A critical role for the cytoplasmic domain of the granulocyte-macrophage colony-stimulating factor α receptor in mediating cell growth. Cell Growth Differ. **4:**523–531.

24a. **Sakamak, K., et al.** Unpublished data.

25. **Sakamaki, K., I. Miyajima, T. Kitamura, and A. Miyajima.** 1992. 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. **11**:3541–3549.
26. **Sato, N., K. Sakamaki, N. Terada, K. I. Arai, and A. Miyajima.** 1993. Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common β subunit responsible for different signaling. EMBO J. **12**:4181–4189.
27. **Satoh, T., M. Nakafuku, A. Miyajima, and Y. Kaziro.** 1991. Involvement of ras p21 protein in signal-transduction pathways from interleukin 2, interleukin 3, and granulocyte/macrophage colony-stimulating factor, but not from interleukin 4. Proc. Natl. Acad. Sci. USA **88**:3314–3318.
28. **Silvennoinen, O., J. N. Ihle, J. Schlessinger, and D. E. Levy.** 1993. Interferon-induced nuclear signaling by JAK protein tyrosine kinases. Nature (London) **366**:583–585.
29. **Silvennoinen, O., B. Witthuhn, F. W. Quelle, J. L. Cleveland, T. Yi, and J. N. Ihle.** 1993. Structure of the JAK2 protein tyrosine kinase and its role in IL-3 signal transduction. Proc. Natl. Acad. Sci. USA **90**:8429–8433.
30. **Sorensen, P. H., A. L. Mui, S. C. Murthy, and G. Krystal.** 1989. Interleukin-3, GM-CSF, and TPA induce distinct phosphorylation events in an interleukin 3-dependent multipotential cell line. Blood **73**:406–418.
31. **Stahl, N., T. G. Boulton, T. Farruggella, N. Y. Ip, S. Davis, B. A. Witthuhn, F. W. Quelle, O. Silvennoinen, G. Barbieri, S. Pellegrini, J. N. Ihle, and G. D. Yancopoulos.** 1994. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 beta receptor components. Science **263**:92–95.
32. **Stahl, N., and G. D. Yancopoulos.** 1993. The alphas, betas and kinases of cytokine receptor complexes. Cell **74**:587–590.
33. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin 1555. Texas Agricultural Experiment Station, College Station.
34. **Velazquez, L., M. Fellous, G. R. Stark, and S. Pellegrini.** 1992. A protein tyrosine kinase in the interferon alpha/beta signaling pathway. Cell **70**:313–322.
35. **Watling, D., D. Guschin, M. Muller, O. Silvennoinen, B. A. Witthuhn, F. W. Quelle, N. C. Rogers, C. Schindler, G. R. Stark, J. N. Ihle, and I. M. Kerr.** 1993. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-τ signal transduction pathway. Nature (London) **366**:166–170.

35a. **Witthuhn, B., F. W. Quelle, and J. N. Ihle.** Unpublished data.

36. **Witthuhn, B., F. W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J. N. Ihle.** 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following EPO stimulation. Cell **74**:227–236.