

# Transactivation of gp130 in Myeloma Cells<sup>1</sup>

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**Receptor transactivation, i.e., interaction between unrelated receptor systems, is a growing theme in cytokine and growth factor signaling. In this study we reveal for the first time the ability of IFN- $\alpha$  to transactivate gp130 in myeloma cells. An epidermal growth factor receptor/gp130 chimeric receptor previously shown by us to transactivate endogenous gp130, provided a complementary tool to study the underlying mechanisms of receptor cross-talk. Further analysis revealed that transactivation of gp130 by IFN- $\alpha$  did not require the extracellular or *trans*-membrane domain of gp130. Moreover, transactivation of gp130 was critically dependent upon Janus kinase activation by the initiating receptor and correlated with rapid and sustained Janus kinase 1 and tyrosine kinase (Tyk) 2 tyrosine phosphorylation. Finally, transactivation of gp130 may be a common theme in myeloma cells, perhaps providing a mechanism for enhanced or qualitatively distinct cellular responses to specific stimuli. *The Journal of Immunology*, 2003, 170: 3717–3723.**

Cytokines and cytokine receptors are classified on the basis of structural similarities and mechanism of action. Many cytokine receptor complexes are comprised of multiple distinct subunits, and ligand-mediated activation is critically dependent upon dimerization or high order oligomerization of receptor subunits. Certain receptor subunits, such as the common  $\gamma$ -chain, the common  $\beta$  chain, and gp130, are used by multiple ligands within the same cytokine subfamily. Specifically, gp130 is a common signal transducing receptor subunit used by the IL-6 cytokine subfamily, including IL-6, IL-11, leukemia inhibitory factor, oncostatin M (OSM),<sup>3</sup> ciliary neurotrophic factor, cardiotrophin-1, and novel neurotrophin-1 (1, 2).

The IL-6R is similar to other cytokine receptors in that it lacks intrinsic kinase activity, relying instead upon the activity of non-receptor Janus kinase (Jak) tyrosine kinases (Tyk). Jak1, Jak2, and Tyk2 constitutively associate with the membrane-proximal Box1 region in gp130 (3–7). Ligand binding to the IL-6R (gp80) triggers homodimerization of gp130, which is believed to result in the juxtaposition and cross-phosphorylation of Jaks. Activated Jaks subsequently phosphorylate tyrosine residues within the cytoplasmic tail of gp130, thereby providing docking sites for Src homology 2 domain-containing molecules, such as SH2-containing protein tyrosine phosphatase and STAT factors, and initiating downstream signaling pathways (8–13). The IL-6 cytokine subfamily has been shown to mediate numerous biological functions, including cellular differentiation, proliferation, and survival (9, 14–17).

IFN- $\alpha$ , a type II cytokine, signals through a dimeric receptor complex composed of two related subunits designated IFNAR-1

and IFNAR-2. Whereas IFNAR-1 alone does not bind IFN- $\alpha$  and IFNAR-2 only binds ligand with low affinity, an IFNAR-1/IFNAR-2 heterodimer binds IFN- $\alpha$  with high affinity. Similarly to gp130, the IFN- $\alpha$  receptor lacks intrinsic kinase activity and instead uses Jak1 and Tyk2 to initiate signal transduction. Ligand-induced dimerization of IFNAR-1 and IFNAR-2 allows juxtaposition and activation of the associated Jaks, leading to activation of downstream signaling pathways (e.g., STAT1, -2, and -3) (18, 19). Although IFN- $\alpha$  and IL-6 are both able to activate the Jak/STAT pathway, it is interesting to note that each cytokine has unique biological effects, e.g., IFN- $\alpha$ , but not IL-6, can induce antiviral responses. These differences are believed to reflect signaling events that are unique to each receptor complex.

Receptors classified in different families are classically thought to function independently, with potential interaction(s) only occurring downstream between amplifying or competing signal transduction intermediates (20–23). This paradigm has been challenged in the last decade by growing evidence of receptor cross-talk, or transactivation (22–33). Rather than signaling independently, unrelated receptors can interact at the level of the receptors themselves, either via a direct physical interaction or in an indirect manner via an associated kinase (e.g., Jaks). Early evidence of receptor transactivation was provided by the discovery that growth hormone (GH) was capable of stimulating tyrosine phosphorylation of the epidermal growth factor receptor (EGFR) tyrosine kinase, ErbB1, in mouse liver. Cross-talk between the GH receptor and ErbB1 was facilitated by Jak2 tyrosine kinase activity and was essential for GH-induced mitogen-activated protein kinase (MAPK) activation (33). In a separate study, Qui et al. (29) demonstrated receptor cross-talk between the IL-6R and the EGFR family member, ErbB3, in the prostate carcinoma cell line, LNCAP. Thus, IL-6 stimulated the physical association of gp130 with ErbB2, which resulted in tyrosine phosphorylation of ErbB3 and subsequent MAPK activation. In a somewhat similar manner, EGF was shown to enhance OSM-induced inhibition of cellular proliferation by the breast carcinoma cell line, MCF-7 (24). In this example, however, gp130 was shown to be constitutively associated with both ErbB2 and ErbB3, and EGF stimulation resulted in tyrosine phosphorylation of gp130.

In addition to IL-6/gp130-mediated transactivation of the ErbB receptor family, there is also evidence of gp130 cross-talk with other receptor families. Thus, additional studies have suggested a potential interaction between the IFN receptor ( $\alpha/\beta$ ,  $\gamma$ ) families

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<sup>3</sup> Abbreviations used in this paper: OSM, oncostatin M; MAPK, mitogen activated protein kinase; EGFR, epidermal growth factor receptor; GH, growth hormone; IFNR, IFN- $\gamma$  receptor; IGF-I, insulin-like growth factor I; Jak, Janus kinase; TM, transmembrane; Tyk, tyrosine kinase.

and the gp130 family of cytokine receptors. Strobl et al. (34) showed that both OSM and IL-6 could induce phosphorylation of the IFN- $\gamma$  receptor (IFN- $\gamma$ R1) in the human fibrosarcoma cell line 2fTGH. Finally, Mitani et al. (28) demonstrated that maximal IL-6-induced STAT activation in mouse embryonic fibroblast required the presence of IFNAR-1.

In light of the accumulating data supporting the ability of gp130 to cross-communicate with unrelated receptor systems and in an effort to better understand IL-6-stimulated growth of malignant plasma cells, we investigated the potential for receptor transactivation of gp130 in myeloma cells. Our studies provide the first evidence for receptor cross-talk between gp130 and the IFN- $\alpha$  receptor system in myeloma cells. Furthermore, an EGFR/gp130 chimeric receptor facilitated transactivation of gp130 in a similar fashion to IFN- $\alpha$ . Thus, we used these unexpected receptor interactions to characterize the mechanisms responsible for gp130 transactivation in myeloma cells.

## Materials and Methods

### Cell lines, culture medium, and reagents

The KAS-6/1, ANBL-6, and DP-6 myeloma cell lines (35, 36) were derived in our laboratory and are maintained in RPMI 1640, 1 ng/ml IL-6, and 10% FCS. Novartis Pharma (Basel, Switzerland) provided rIL-6. Recombinant EGF was purchased from BioSource (Camarillo, CA). Recombinant IFN- $\alpha$  was purchased from Schering (Kenilworth, NJ). Recombinant insulin-like growth factor I (IGF-I) was purchased from Intergen (Purchase, NY). The B-R3 anti-gp130 Ab (BioSource) and anti-EGFR (Ab-1) Ab (Oncogene, Cambridge, MA) were used for immunoprecipitation, and an anti-c-Myc Ab (Oncogene) was used for immunoprecipitation and immunoblotting. The anti-IFNAR1 Ab used for immunoprecipitation and immunoblotting was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Jak1 (immunoprecipitation), anti-Jak2 (immunoprecipitation and immunoblotting), anti-Tyk2 (immunoprecipitation), anti-gp130 (immunoblotting), and anti-phosphotyrosine (immunoblotting) Abs were purchased from Upstate Biotechnology (Lake Placid, NY). The anti-Jak1, anti-Jak2, and anti-Tyk2 Abs used for immunoblotting were purchased from BD PharMingen (San Diego, CA). Gamma-Bind protein G-Sepharose beads and protein A-Sepharose beads were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). HRP-linked anti-mouse or rabbit IgG secondary Abs and an ECL detection system were purchased from Amersham Pharmacia Biotech.

### EGFR/gp130 constructs and generation of stable transfectants

The EGFR/gp130 chimeric receptor constructs (cloned into the SR $\alpha$  expression vector) were provided by Dr. N. Stahl (Regeneron Pharmaceuticals, Tarrytown, NY) (37). The chimeric receptor constructs were first subcloned into the pCI-Neo expression vector (Promega, Madison, WI). The EGFR transmembrane (TM) chimeric receptor construct was generated as previously described (38). The BOX1 mutant construct was generated by PCR SOEing (39) using the full-length EGFR/gp130 construct as a template and modified primers with point mutations that convert the two proline residues at positions 656 and 658 into glycine residues. The EGFR<sup>TM</sup> and BOX1 constructs were verified by nucleotide sequence analysis before use. Cells were transfected with 40  $\mu$ g of DNA using a square wave electroporator (BTX electroporation system; Genetronics, San Diego, CA) set at 20 ms and 250 V. Bulk transfectants were selected in 250  $\mu$ g/ml G418 (Calbiochem, La Jolla, CA) and sorted for high expression of the EGFR/gp130 chimera by FACS. Stable transfectants were maintained in growth medium supplemented with 250  $\mu$ g/ml G418.

### Whole cell lysate preparation, immunoprecipitation, and Western blot analysis

For immunoprecipitation of gp130, IFNAR1, the chimeric receptor, Jak1, Jak2, and Tyk2, cells were starved overnight in RPMI plus 0.5% BSA. Cells ( $10 \times 10^6$ ) were stimulated with 50 ng/ml IL-6, 100 ng/ml EGF, 10,000 U/ml IFN- $\alpha$ , and 100 ng/ml IGF-I for the designated time at 37°C and lysed in cold lysis buffer as previously described (38). Lysates were cleared of insoluble material by centrifugation for 10 min at 14,000 rpm. Two micrograms of anti-gp130 or 1  $\mu$ g anti-EGFR Ab prebound to  $\gamma$ -Bind protein G-Sepharose beads, or 1  $\mu$ g anti-Jak1, 1  $\mu$ g anti-Tyk2, 1  $\mu$ g anti-Jak2, or 1  $\mu$ g anti-IFNAR1 prebound to protein A-Sepharose beads were

incubated with cellular lysates for a minimum of 3 h. The immunoprecipitates were washed three times in lysis buffer and analyzed by SDS-PAGE.

### DNA synthesis assay

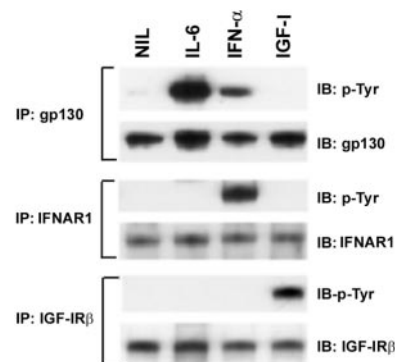
Myeloma cells were washed twice to remove IL-6 present in the culture medium and resuspended in RPMI plus 0.5% BSA overnight. Cells were cultured in 96-well, flat-bottom microtiter plates (Costar, Cambridge, MA) at a density of  $2.5 \times 10^4$  cells/well and in a final volume of 200  $\mu$ l. Cultures were conducted in triplicate in the presence of 1 ng/ml IL-6 or 1000 U/ml IFN- $\alpha$  alone or in combination for 3 days at 37°C in the presence of 5% CO<sub>2</sub>. Cultures were pulsed with 1  $\mu$ Ci of tritiated thymidine (<sup>3</sup>H]TdR; 5.0 Ci/mmol; Amersham Pharmacia Biotech) for the last 18 h of culture. [<sup>3</sup>H]TdR levels were determined using a scintillation counter (Beckman, Palo Alto, CA).

## Results

### IFN- $\alpha$ induces phosphorylation of gp130 in myeloma cells

Receptor transactivation has the potential to amplify or diversify downstream responsiveness. Because IL-6 is known to play a central role in the biology of myeloma cells, we wished to determine whether other cytokines/growth factors could transactivate gp130 using the IL-6 growth-responsive myeloma cell line, KAS-6/1. Although these cells are maintained in IL-6, we have previously reported that a number of other cytokines also stimulate DNA synthesis in this cell line (35). We first screened a panel of cytokines and growth factors for their ability to induce tyrosine phosphorylation of the IL-6R subunit gp130 (results not shown). Our studies revealed that IFN- $\alpha$ -stimulated significant tyrosine phosphorylation of gp130.

To further characterize the interaction between the IFN- $\alpha$  and IL-6 receptor families we assessed the ability of IL-6 to induce phosphorylation of IFNAR1, a subunit of the IFN- $\alpha$  receptor complex. The KAS-6/1 cell line was stimulated with IL-6, IFN- $\alpha$ , or IGF-I. As shown in Fig. 1, IL-6, IFN- $\alpha$ , and IGF-I stimulation induced tyrosine phosphorylation of gp130, IFNAR1, and IGF-IR, respectively. Of interest, IFN- $\alpha$  stimulation induced tyrosine phosphorylation of the unrelated receptor subunit, gp130, whereas IGF-I stimulation did not (Fig. 1, upper panel, lanes 3 vs 4). Although IFN- $\alpha$ -induced activation of gp130, IL-6 did not transactivate IFNAR1 (Fig. 1, middle panel, lane 2) or IFNAR2 (results not shown). Thus, cross-talk between the IFN- $\alpha$  receptor and gp130 was unidirectional. The interaction between IFNAR and gp130 was most likely indirect or transient, as we were unable to coimmunoprecipitate gp130 with either IFNAR1 or IFNAR2 after IFN- $\alpha$  stimulation (results not shown).

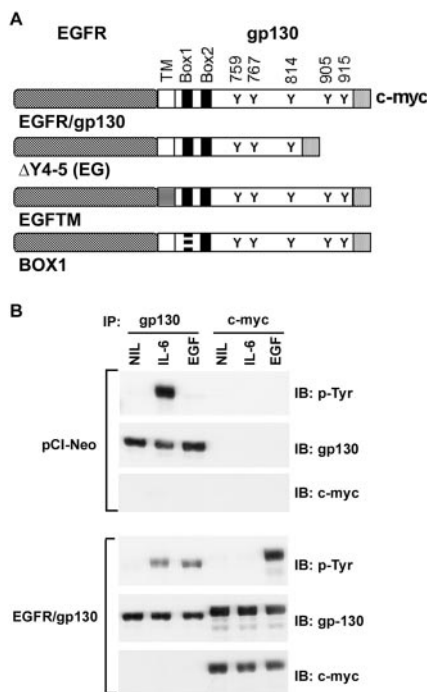


**FIGURE 1.** IFN- $\alpha$  transactivates gp130 in KAS-6/1 myeloma cells. KAS-6/1 cells were stimulated for 10 min with IL-6, IFN- $\alpha$ , or IGF-I. Cell lysates were incubated with anti-gp130 (B-R3), anti-IFNAR1, or anti-IGF-IR $\beta$  to immunoprecipitate gp130, IFNAR1, and IGF-IR $\beta$ , respectively. Precipitated proteins were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, and immunoblotted with the designated Abs.

### Transactivation of gp130 by an EGFR/gp130 chimeric receptor

We have previously shown that EGFR/gp130 chimeric receptors composed of the EGFR extracellular domain, the gp130 TM domain, and the full-length or truncated gp130 cytoplasmic domain were unexpectedly capable of activating endogenous gp130 in both KAS-6/1 and ANBL-6 myeloma cell lines (38). This observation seemed to parallel the ability of IFN- $\alpha$  to transactivate gp130, suggesting that transactivation of gp130 may be a common theme in myeloma cell signal transduction. Thus, stimulation through either the IFNAR or EGFR/gp130 chimeric receptor provided a model system to further elucidate the general principles that govern transactivation of gp130 in myeloma cells.

A schematic of the chimeric receptors used in this study is shown in Fig. 2A. Fig. 2B demonstrates the ability of the EGFR/gp130 chimeric receptor to transactivate endogenous gp130. Endogenous gp130 was immunoprecipitated using an Ab directed against the extracellular domain of the receptor (Fig. 2B, lanes 1–3), and the EGFR/gp130 chimeric receptor was immunoprecipitated using an Ab against the engineered c-Myc tag (Fig. 2B, lanes 4–6). In KAS-6/1 cells expressing EGFR/gp130, IL-6 stimulated phosphorylation of endogenous gp130, and EGF induced phosphorylation of the chimeric receptor. Although KAS-6/1 cells expressing empty vector (pCI-Neo) showed no response to EGF be-

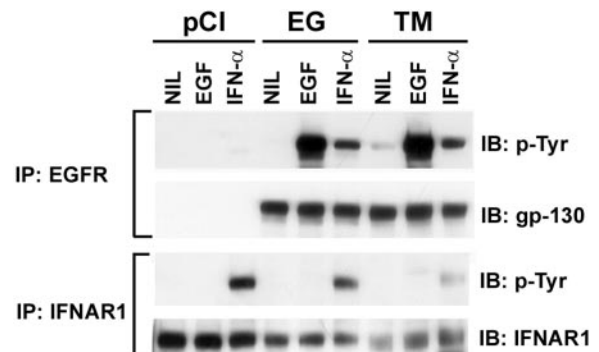


**FIGURE 2.** Gp130 transactivation by an EGFR/gp130 chimeric receptor. *A*, The EGFR/gp130 chimeric receptors are composed of the extracellular domain of the EGFR and the TM and cytoplasmic domains of gp130 (residues 620–918). The  $\Delta$ Y4–5 chimeric receptor lacks residues 905–918 of the gp130 cytoplasmic tail. The EGFRTM chimeric receptor contains the TM region of EGFR (residues 620–644). The BOX1 chimeric receptor was derived from EGFR/gp130, containing glycine substitutions for the two proline residues at positions 656 and 658 in the Box1 motif. A carboxyl-terminal c-Myc tag provides a target for immunoprecipitation. *B*, KAS-6/1 transfectants expressing empty vector (pCI) or full-length chimeric receptor (EGFR/gp130) were stimulated for 10 min with IL-6 or EGF. Cell lysates were incubated with anti-gp130 (B-R3) or anti-c-Myc Abs to immunoprecipitate endogenous gp130 or the chimeric receptor, respectively. Precipitated proteins were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, and immunoblotted with the designated Abs.

cause they do not express endogenous EGF receptors (38), EGFR/gp130 transfectants exhibited substantial tyrosine phosphorylation of endogenous gp130 upon EGF stimulation. In previous studies we have shown that this interaction occurred even when using an EGFR/gp130 truncation mutant containing only 116 aa of the gp130 cytoplasmic tail (38). As observed previously (38), communication between EGFR/gp130 and endogenous gp130 was unidirectional, since IL-6 stimulation did not result in tyrosine phosphorylation of the chimeric receptor (Fig. 2B, lower panel, lane 5).

### Transactivation of gp130 does not require the extracellular or TM domains of gp130

To determine the structural requirements for gp130 transactivation, we next assessed the ability of IFN- $\alpha$  to induce tyrosine phosphorylation of the EGFR/gp130 chimeric receptor, which maintains only the TM and cytoplasmic tail of gp130. Since the transfectants expressing the full-length EGFR/gp130 chimeric receptor often displayed high basal phosphorylation of endogenous gp130 (38), we used the  $\Delta$ Y4–5 chimeric receptor, which lacks C-terminal residues 905–918 (Fig. 2A, EG). KAS-6/1 cells expressing either empty vector (pCI) or the EGFR/gp130  $\Delta$ Y4–5 (EG) chimeric receptor were stimulated with EGF or IFN- $\alpha$ . The chimeric receptor and endogenous IFNAR1 were immunoprecipitated, and phosphorylation status was assessed by immunoblot (Fig. 3). As expected, IFN- $\alpha$  stimulation resulted in phosphorylation of IFNAR1 in both pCI and EG transfectants (Fig. 3, panel 3, lanes 3 and 6), while EGF stimulation induced tyrosine phosphorylation of the chimeric receptor (Fig. 3, panel 1, lane 5). Moreover, IFN- $\alpha$  stimulation resulted in tyrosine phosphorylation of the chimeric receptor (Fig. 3, panel 1, lane 6). This interaction was unidirectional, as EGF stimulation did not induce tyrosine phosphorylation of IFNAR1 (Fig. 3, panel 3, lane 5) or IFNAR2 (results not shown). Thus, just as IFN- $\alpha$  stimulation resulted in tyrosine phosphorylation of endogenous, wild-type gp130, IFN- $\alpha$  stimulation also triggered phosphorylation of the EGFR/gp130 chimeric receptor, which lacks the extracellular domain of gp130. To determine the TM requirements for IFN- $\alpha$  transactivation of gp130, we next assessed the phosphorylation status of the EGFRTM chimeric receptor, in which the TM domain of gp130 was replaced by the corresponding domain of EGFR (Fig. 3, lanes 7–9). KAS-6/1 transfectants expressing the EGFRTM chimeric receptor were



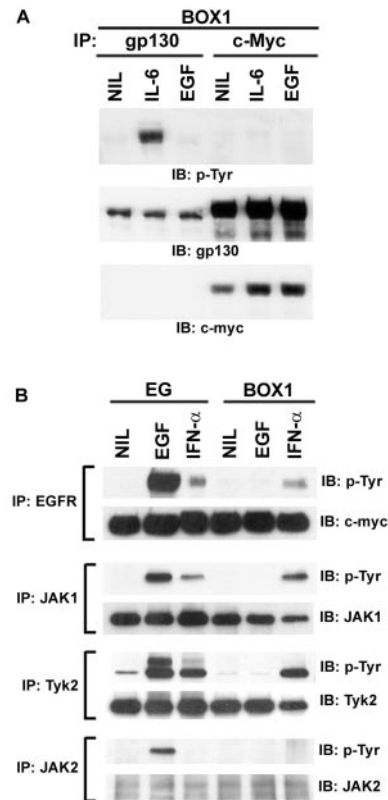
**FIGURE 3.** IFN- $\alpha$  transactivation of the EGFR/gp130 chimeric receptor. KAS-6/1 transfectants expressing empty vector (pCI), the EG chimeric receptor, or the EGFRTM chimeric receptor were stimulated for 10 min with EGF or IFN- $\alpha$ . Cell lysates were incubated with anti-EGFR or anti-IFNAR1 Abs to immunoprecipitate the chimeric receptor or IFNAR1, respectively. Precipitated proteins were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, and immunoblotted with the designated Abs.

stimulated with EGF or IFN- $\alpha$ . Immunoprecipitation and immunoblotting techniques revealed that the EGFR<sup>TM</sup> chimeric receptor was tyrosine phosphorylated after both EGF and IFN- $\alpha$  stimulation. These data suggest that transactivation of gp130 does not require either the extracellular or TM domain of gp130 and, instead, may be mediated by an intracellular mechanism.

*Transactivation of gp130 requires Jak activity by the initiating receptor (ligand-dependent), but not by the receiving receptor (ligand-independent)*

We were next interested in exploring the role of Jak activation in both models of gp130 transactivation. As described above, the EGFR/gp130 chimeric receptor can act as either an initiator or as a target of transactivation and could be strategically modified allowing us to further characterize the mechanism of gp130 transactivation. Therefore, to investigate the role of Jak kinases in gp130 transactivation we disrupted the Box1 motif of the EGFR/gp130 cytoplasmic tail (5). The two critical proline residues (Pro<sup>656</sup> and Pro<sup>658</sup>) within the Box1 motif were exchanged for glycine residues in the full-length EGFR/gp130 chimeric receptor (Fig. 2A, BOX1). Using KAS-6/1 transfectants, which stably express the BOX1 chimeric receptor, we first assessed the ability of the mutant EGFR/gp130 chimeric receptor to stimulate tyrosine phosphorylation of endogenous gp130. As expected, disrupting the Box1 domain of the EGFR/gp130 abrogated EGF-induced phosphorylation of the chimeric receptor (Fig. 4A, upper panel, lane 6). Furthermore, whereas IL-6 induced phosphorylation of endogenous gp130 in the Box1 transfectants (Fig. 4A, lane 2), EGF stimulation no longer transactivated endogenous gp130 (Fig. 4A, lane 3). Importantly, Jak1, Tyk2, and Jak2 were not activated by the BOX1 chimeric receptor in response to EGF (Fig. 4B, panels 2–4). These data suggest that Jak activation by the initiating, ligand-dependent, receptor is required for transactivation of gp130.

We next wanted to determine whether Jak activation by the receiving, ligand-independent receptor, i.e., gp130, was necessary for tyrosine phosphorylation. To accomplish this, we assessed the ability of IFN- $\alpha$  to induce phosphorylation of the BOX1 mutant EGFR/gp130 chimeric receptor. KAS-6/1 cells expressing either the EG or BOX1 chimeric receptor were stimulated with EGF or IFN- $\alpha$  (Fig. 4B). As expected, EGF induced tyrosine phosphorylation of the EG, but not the BOX1 chimeric receptor (Fig. 4B, upper panel, lanes 2 and 5). In contrast, IFN- $\alpha$  stimulation resulted in tyrosine phosphorylation of both the EG and BOX1 chimeric receptors (Fig. 4B, upper panel, lanes 3 and 6). To determine the role of each of the Jak kinases known to be activated by gp130, we next assessed Jak1, Tyk2, and Jak2 tyrosine phosphorylation following cytokine stimulation. Although the EG chimeric receptor facilitated Jak1, Tyk2, and Jak2 activation, disruption of the Box1 motif eliminated the ability of the BOX1 chimeric receptor to activate all three kinases following EGF stimulation (Fig. 4B, panels 2–4). IFN- $\alpha$  stimulation resulted in tyrosine phosphorylation of Jak1 and Tyk2, but not Jak2, in KAS-6/1 transfectants expressing either the EG or BOX1 chimeric receptors (Fig. 4B, lanes 3 and 6, panels 2–4). Thus, even though the BOX1 chimeric receptor lacked the ability to activate all three Jak kinases, it was readily phosphorylated following stimulation with IFN- $\alpha$ . These data suggest that Jak activation by the initiating receptor, but not the receiving receptor, is essential for transactivation. Furthermore, the IFN- $\alpha$  receptor and the EGFR/gp130 chimeric receptor were both capable of activating Jak1 and Tyk2, suggesting that both kinases may be involved in gp130 transactivation.



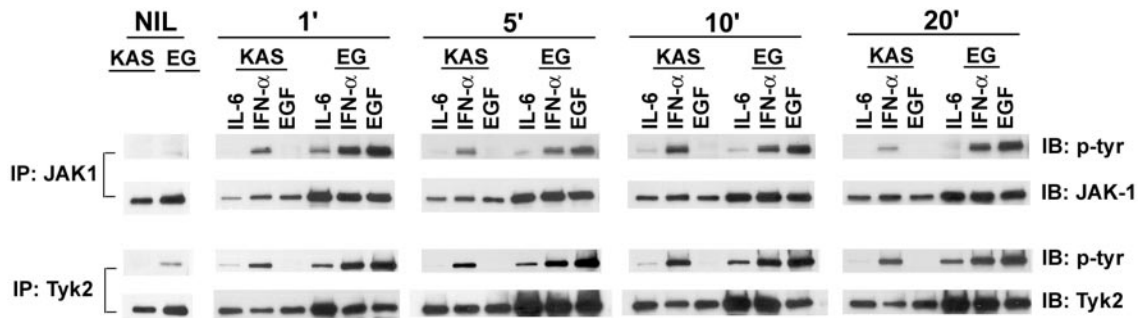
**FIGURE 4.** Jak activation is required for receptor transactivation. *A*, The KAS-6/1 BOX1 transfectants were stimulated for 10 min with IL-6 or EGF. Cell lysates were incubated with anti-gp130 or anti-c-Myc Abs to immunoprecipitate endogenous gp130 or the BOX1 chimeric receptor, respectively. *B*, The KAS-6/1 transfectants expressing either the EG or the BOX1 chimeric receptor were stimulated for 10 min with EGF or IFN- $\alpha$ . Cell lysates were incubated with anti-EGFR, anti-Jak1, anti-Tyk2, and anti-Jak2 Abs to immunoprecipitate the chimeric receptor or each Jak kinase, respectively. Precipitated proteins were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, and immunoblotted with the designated Abs.

*Enhanced phosphorylation of Jak1 and Tyk2 correlates with gp130 transactivation*

As shown above, Jak activation appeared to be critical for transactivation of gp130. To further address the role of Jak phosphorylation in gp130 transactivation, we assessed Jak1 and Tyk2 activation following cytokine stimulation of the parental KAS-6/1 cells and the KAS-6/1 EG chimeric receptor transfectants (Fig. 5). Jak1 and Tyk2 tyrosine phosphorylation status was analyzed following stimulation of KAS-6/1 (lanes 1–3) or EG cells (lanes 4–6) with IL-6, IFN- $\alpha$ , or EGF for 1, 5, 10, and 20 min. The levels of Jak1 and Tyk2 activation following IL-6 stimulation were modest compared with levels mediated by IFN- $\alpha$  or EGF. Indeed, IFN- $\alpha$  induced rapid and sustained activation of both Jak family members in both parental KAS-6/1 and the EG transfectants, attaining high level phosphorylation by 1 min that was maintained as long as 20 min. Similarly, EGF stimulation of the EG transfectants resulted in robust and sustained Jak1 and Tyk2 activation. Thus, enhanced Jak activation correlated with gp130 transactivation in both models of receptor cross-talk.

*IFN- $\alpha$ -induced phosphorylation of gp130 is not unique to the KAS-6/1 myeloma cell line*

To address the possibility that this phenomenon was unique to the KAS-6/1 cell line, we next assessed the potential for gp130 transactivation in the ANBL-6 and DP-6 myeloma cell lines. Analysis

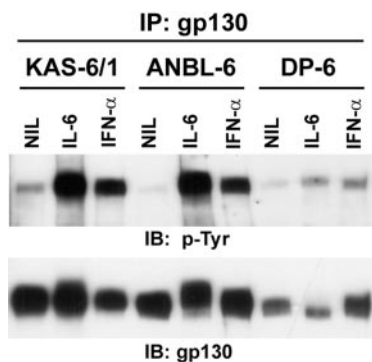


**FIGURE 5.** Both the IFNAR and the EGFR/gp130 chimeric receptor support enhanced Jak1 and Tyk2 phosphorylation. Parental KAS-6/1 cells or EG chimeric receptor transfectants were stimulated for 1, 5, 10, or 20 min with IL-6, IFN- $\alpha$ , or EGF. Cell lysates were incubated with anti-Jak1 or anti-Tyk2 Abs to immunoprecipitate Jak1 or Tyk2, respectively. Precipitated proteins were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, and immunoblotted with the designated Abs.

of the tyrosine phosphorylation status of gp130 revealed that IFN- $\alpha$  stimulation also transactivated gp130 in both ANBL-6 and DP-6 myeloma cell lines (Fig. 6). This observation was unexpected because of the striking differences between the ability of IFN- $\alpha$  and IL-6 to function as growth factors for these three cell lines. Thus, similar to our previous findings (40), Table I displays the results of a representative experiment demonstrating that IFN- $\alpha$  stimulation alone promotes atypical cell growth in the KAS-6/1 cell line, whereas it suppresses ANBL-6 cell growth and is ineffective in directly enhancing DP-6 cell proliferation. Of note, however, despite direct stimulation of KAS-6/1 cell proliferation, IFN- $\alpha$  stimulation uniformly attenuated the magnitude of IL-6 responsiveness in all three cell lines. This observation may be explained by the ability of IFN- $\alpha$  to transactivate gp130. Collectively, these data suggest that cross-talk between IFNAR and gp130 may play a significant role in myeloma cell biology.

**Discussion**

In the present study we have revealed the ability of gp130 to be activated in the absence of direct ligand binding, i.e., independent of IL-6R (gp80) and IL-6. Our studies show for the first time that IFN- $\alpha$  stimulation can induce tyrosine phosphorylation of gp130 in IL-6 growth-responsive myeloma cell lines. Using two models of gp130 transactivation, we specifically focused our studies on defining the mechanisms responsible for gp130 transactivation in myeloma cells.



**FIGURE 6.** Gp130 transactivation by IFN- $\alpha$  is not unique to the KAS-6/1 myeloma cell line. KAS-6/1, ANBL-6, and DP-6 cells were stimulated for 10 min with IL-6 or IFN- $\alpha$ . Cell lysates were incubated with anti-gp130 (B-R3) to immunoprecipitate gp130. Precipitated proteins were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, and immunoblotted with the designated Abs.

Our data suggest that transactivation of gp130 does not require the extracellular or TM domain of gp130. Specifically, IFN- $\alpha$  stimulation resulted in phosphorylation of an EGFR/gp130 chimeric receptor lacking both the TM and extracellular domains of gp130. Thus, transactivation of gp130 seems to be an intracellular phenomenon requiring the presence of only the cytoplasmic tail of gp130. Studies using the EGFR/gp130 chimeric receptor, which lacks a functional Box1 domain, revealed that gp130 transactivation requires Jak activation by the initiating receptor. Furthermore, our studies revealed that gp130 transactivation correlated with enhanced Jak activation. Both IFN- $\alpha$  and EGF induced rapid and sustained high level phosphorylation of Jak1 and Tyk2 compared with IL-6 stimulation. In support of this theory, Yamauchi et al. (33) showed increased phosphorylation levels of Jak2 in response to GH and prolactin, both of which are capable of transactivating ErbB1, while Jak2 activation by IL-6, leukemia inhibitory factor, and IFN- $\gamma$  was dramatically reduced in comparison.

In further support of a role for Jak kinases in gp130 transactivation, previous work by our laboratory revealed that EGFR/gp130  $\Delta$ Y1-5, which maintains only 116 membrane-proximal aa of the gp130 cytoplasmic domain, was capable of phosphorylating endogenous gp130 in the KAS-6/1 cell line (38). Thus, receptor transactivation can occur even in the absence of tyrosine residues known to be essential for the initiation of downstream signal transduction. While Jaks may be responsible for direct phosphorylation of gp130 in response to IFN- $\alpha$ , our data do not eliminate the possibility that Jaks may be acting indirectly. Thus, Jak-dependent phosphorylation of the initiating receptor may be essential for activation of an intermediate tyrosine kinase (e.g., Src (30)), receptor tyrosine kinases (24, 29)), which may, in turn, act directly to transactivate gp130. Although myeloma cells are known to express Src family kinases (8), 4-amino-5(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine, which specifically inhibits Src family kinases (41), had no effect on IFN- $\alpha$ -induced or EGFR/gp130-mediated phosphorylation of gp130 (results not shown).

Table I. Myeloma cell line responsiveness to IFN- $\alpha$ <sup>a</sup>

Cell Line	Stimulation (cpm $\times 10^{-3} \pm$ SEM)			
	Nil	IFN- $\alpha$	IL-6	IL-6 + IFN- $\alpha$
KAS-6/1	0.2 $\pm$ 0.1	11.1 $\pm$ 0.4	98.4 $\pm$ 2.9	33.9 $\pm$ 1.2
DP-6	22.6 $\pm$ 1.1	26.7 $\pm$ 1.5	130.1 $\pm$ 5.0	62.4 $\pm$ 1.5
ANBL-6	5.0 $\pm$ 0.2	0.2 $\pm$ 0.1	71.1 $\pm$ 4.3	2.6 $\pm$ 0.1

<sup>a</sup> Myeloma cells were cultured at  $2.5 \times 10^4$  cells/well in the presence or the absence of the indicated stimuli. DNA synthesis was measured on day 3. Results are representative of five independent experiments.

While Jak activation seems to play a critical role in gp130 transactivation, the ability of specific receptors to interact is probably determined by numerous factors. Although EGF stimulation through EGFR/gp130 induced enhanced Jak1 and Tyk2 activation, EGF could not transactivate the IFNAR (Fig. 3). Similarly, IFN- $\alpha$  could not transactivate IGF-IR (Fig. 1). Thus, enhanced Jak1 or Tyk2 activation alone is not sufficient to mediate receptor transactivation. Instead, the ability of specific receptors to interact may be determined by numerous factors, including kinase specificity, receptor structure, and receptor localization. Studies in mouse embryonic fibroblasts suggest that colocalization of IFNGR and gp130 with IFNAR1 within caveolae facilitated cross-communication (28, 31). Additionally, gp130 was found to localize within the caveolae of human kidney epithelial cells (42). While the presence of caveolae in lymphocytes remains controversial (43–45), the role of lipid rafts in lymphocytes is well documented. Essential components of the cytokine signaling system, including gp130, IFNGR, and STAT factors (46), are thought to be localized to such membrane microdomains. The ability of a receptor to be transactivated may also depend upon its surface expression level and/or availability. Under ideal conditions where Jak activation is enhanced and receptor structure is not limiting, the target receptor must be easily accessible. Importantly, gp130 expression levels are commonly increased in myeloma cells (47), perhaps providing an easy target for transactivation.

Our studies reveal that IFN- $\alpha$  is capable of transactivating gp130 in three independent, IL-6 growth-responsive myeloma cell lines. While these cell lines are differentially responsive to IFN- $\alpha$ , all showed IFN- $\alpha$ -mediated down-regulation of the IL-6 response. For example, while KAS-6/1 cells proliferate in response to both IL-6 and IFN- $\alpha$ , costimulation results in a diminished, rather than heightened, response. This observation may be explained by the observed cross-talk between the IFNAR and gp130. Thus, IFNAR and IL-6R may compete for usage of gp130. While IFN- $\alpha$  stimulation does not induce down-regulation of gp130 (results not shown), transactivation of gp130 by IFN- $\alpha$  may render gp130 refractory to IL-6 stimulation. Alternatively, IFN- $\alpha$  may sequester gp130 to a subcellular location that is not accessible by the IL-6R.

The data presented above provide an essential foundation for future delineation of the biological consequences of gp130 transactivation in myeloma cells. In the majority of receptor cross-talk studies, transactivation via tyrosine phosphorylation positively contributes to the cellular response. For example, GH-induced tyrosine phosphorylation of the EGFR facilitates ERK activation (33). Furthermore, platelet-derived growth factor-induced tyrosine phosphorylation of EGFR is required for platelet-derived growth factor-mediated migration (48). Thus, in general, transactivation is not simply a bystander effect, but plays an important role in the cellular response. Of interest, recent studies suggest that transactivation may lead to only partial activation of the target receptor. Moro et al. (49) revealed that integrin-mediated transactivation of EGFR resulted in a unique phosphorylation pattern distinct from that achieved via direct EGF stimulation.

Similarly, our studies suggest that gp130 transactivation by IFN- $\alpha$  may be qualitatively distinct from direct ligand-induced activation. Gp130 has been shown to be internalized in both a ligand-independent (50, 51) and a ligand-dependent fashion (52–55). While IL-6 stimulation induces activation and down-regulation of gp130 in the KAS-6/1, ANBL-6, and DP-6 myeloma cell lines, gp130 is not internalized following IFN- $\alpha$  stimulation despite robust tyrosine phosphorylation (results not shown). Of note, serine phosphorylation is thought to play a role in gp130 internalization (53, 56). Although gp130 is tyrosine phosphorylated following IFN- $\alpha$  stimulation, serine phosphorylation may not occur as a re-

sult of transactivation. These observations suggest that gp130 is not fully activated following IFN- $\alpha$  stimulation. Instead, specific phosphorylated tyrosine residues within the cytoplasmic tail of gp130 may simply provide a site of signal initiation allowing diversification or enhancement of the initial IFN- $\alpha$  signal.

Signal diversification via receptor transactivation is best exemplified by the documented interaction between IFNGR and IFNAR. While IFNGR is capable of activating STAT1 directly via a STAT1 binding motif within the cytoplasmic tail of IFNGR1, the IFNGR subunits lack STAT2 docking sites. Takaoka et al. (31) demonstrated that IFN- $\gamma$ -induced STAT2 activation required the presence of the IFNAR1 subunit that houses direct binding sites for STAT2. Thus, transactivation of IFNAR1 by IFN- $\gamma$  facilitated expanded STAT activation and enhanced antiviral activity. Similarly, while IFN- $\alpha$  has been shown to activate STAT1, STAT2, and STAT3, the IFNAR subunits lack the STAT3 consensus binding site. Furthermore, STAT3 was shown to be activated by IFN- $\alpha$  even in the absence of all tyrosine residues within the cytoplasmic tail of the receptor (57). In contrast, gp130 houses four direct STAT3 docking sites (37, 58). Thus, gp130 transactivation by IFN- $\alpha$  in myeloma cell lines may provide an indirect mechanism for STAT3 activation by IFN- $\alpha$ .

Receptor transactivation is a growing theme in the world of signal transduction. The mechanisms responsible for the ability of distinct receptor systems to interact at the level of the receptors themselves remain incompletely defined. Importantly, such receptor interactions may encourage altered cellular responses, potentially contributing to tumorigenesis. Furthermore, because malignant cells often display atypical expression of a variety of signaling receptors, it is possible that receptor transactivation may underlie accompanying alterations in cytokine responsiveness, resulting in increased cell growth and/or resistance to apoptosis. Because of these considerations, it is essential to first define the pathways of receptor cross-talk in tumor cells as a necessary step in designing new and more effective therapies. These studies represent an important advance in our knowledge of gp130 activation in myeloma, a human malignancy in which IL-6 is known to play a pivotal role.

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