Cell, Vol. 74, 237–244, July 30, 1993, Copyright © 1993 by Cell Press

Identification of JAK2 As a Growth Hormone Receptor-Associated Tyrosine Kinase

Lawrence S. Argetsinger,* George S. Campbell,* Xianjie Yang,† Bruce A. Witthuhn,‡ Olli Silvennoinen,‡ James N. Ihle,‡ and Christin Carter-Su*
*Department of Physiology
University of Michigan Medical School
Ann Arbor, Michigan 48109
†Department of Genetics
Harvard Medical School
Boston, Massachusetts 02115
‡Department of Biochemistry
St. Jude Children's Research Hospital
Memphis, Tennessee 38105

Summary

Growth hormone receptor (GHR) forms a complex with a tyrosine kinase, suggesting involvement of a ligand-activated tyrosine kinase in intracellular signaling by growth hormone (GH). Here we identify JAK2, a nonreceptor tyrosine kinase, as a GHR-associated tyrosine kinase. Immunological approaches were used to establish GH-dependent complex formation between JAK2 and GHR, activation of JAK2 tyrosine kinase activity, and tyrosyl phosphorylation of both JAK2 and GHR. The JAK2-GHR and JAK2-erythropoietin receptor interactions described here and in the accompanying paper provide a molecular basis for involvement of tyrosyl phosphorylation in physiological responses to these ligands and suggest a shared signaling mechanism among members of the cytokine/hematopoietin receptor family.

Introduction

Although the ability of growth hormone (GH) to promote growth and regulate metabolism has been known for many years (Cheek and Hill, 1974; Davidson, 1987), the molecular mechanism by which GH binding to its receptor elicits its diverse responses has remained an enigma. New insight into GH signaling mechanisms was recently provided by the demonstration that a tyrosine kinase is present in a complex with GH receptor (GHR) prepared from GH-treated cells (Carter-Su et al., 1989; Stred et al., 1992; Wang et al., 1992). Additional studies in 3T3-F442A cells showing rapid GH-dependent tyrosyl phosphorylation of multiple proteins, tyrosyl phosphorylation of microtubule-associated protein kinases, and stimulation of microtubule-associated protein kinase activity, as well as the inhibition of these actions by inhibitors of the GHR-associated tyrosine kinase (Campbell et al., 1993), suggest a central role for a GHR-associated tyrosine kinase in signaling by GH. Recently, a nonreceptor tyrosyl phosphorylated 121 kd protein was identified in a kinase-active GH–GHR preparation (Wang et al., 1993). Since autophosphorylation is often a manifestation of an activated kinase, it was hypothesized that this 121 kd phosphoprotein is the GHR-associated kinase.

In this study, we identify JAK2, a 130 kd tyrosine kinase (Silvennoinen et al., 1993; Harpur et al., 1992), as a GHR-associated kinase. JAK2 is a member of the recently described Janus family of tyrosine kinases consisting of JAK1, JAK2, and Tyk2. In addition to having a kinase domain, these proteins are characterized by the presence of a second kinase-like domain and the absence of Src homology 2 (SH2), SH3, and membrane-spanning domains (Wilks et al., 1991; Firmbach-Kraft et al., 1990; Harpur et al., 1992; Silvennoinen et al., 1993). Members of the JAK family were thought to be good candidates for the GHR-associated tyrosine kinase because of their size (M_r of ~130,000), their presence in GH-responsive tissues, and the ability of Tyk2 to serve as a signaling molecule for the interferon α/β (IFN-α/β) receptor, a receptor distantly related to GHR (Harpur et al., 1992; Silvennoinen et al., 1993; Wilks et al., 1991; Firmbach-Kraft et al., 1990; Bazan, 1990; Velazquez et al., 1992). Here we show that GH binding promotes association of JAK2 with GHR, activation of JAK2, and tyrosyl phosphorylation of both JAK2 and GHR. The identification of JAK2 as a signaling molecule early in the GHR signal transduction pathway provides important insight into signaling by GHR and into the function of JAK2. Work presented in the accompanying paper (Witthuhn et al., 1993 [this issue of *Cell*]) indicates that JAK2 also associates with the receptor for erythropoietin (EPO), and other data indicate that at least four other members of the cytokine/hematopoietin receptor family (receptors for interleukin [IL]-3), granulocyte-macrophage colony-stimulating factor [GM-CSF], granulocyte colony-stimulating factor [G-CSF], and prolactin) and the more distantly related IFN-γ receptor activate JAK2 (Silvennoinen et al., 1993; Witthuhn et al., 1993; O. S. et al., unpublished data; G. S. C. et al., unpublished data). It therefore seems likely that the JAK2–GHR and JAK2–EPO receptor interactions shown in this paper and by Witthuhn et al. (1993) will serve as prototypes for signaling through many members of this large receptor superfamily.

Results

GH Stimulates Tyrosyl Phosphorylation of JAK2

On the basis of previous studies establishing the existence of a GHR-associated tyrosine kinase (Carter-Su et al., 1989; Stred et al., 1992; Wang et al., 1993; Campbell et al., 1993), the GHR-associated tyrosine kinase would be expected: first, to be a protein of ~120 kd; second, to be tyrosyl phosphorylated in response to GH; third, to be present in a complex with GHR; and fourth, to exhibit increased activity in response to GH.

JAK2 is a tyrosine kinase of the correct size (M_r of ~130,000; Silvennoinen et al., 1993) to be the GHR-associated kinase and was therefore tested for its ability to be phosphorylated in response to GH. Solubilized proteins from GH-treated 3T3-F442A fibroblasts were immunopre-

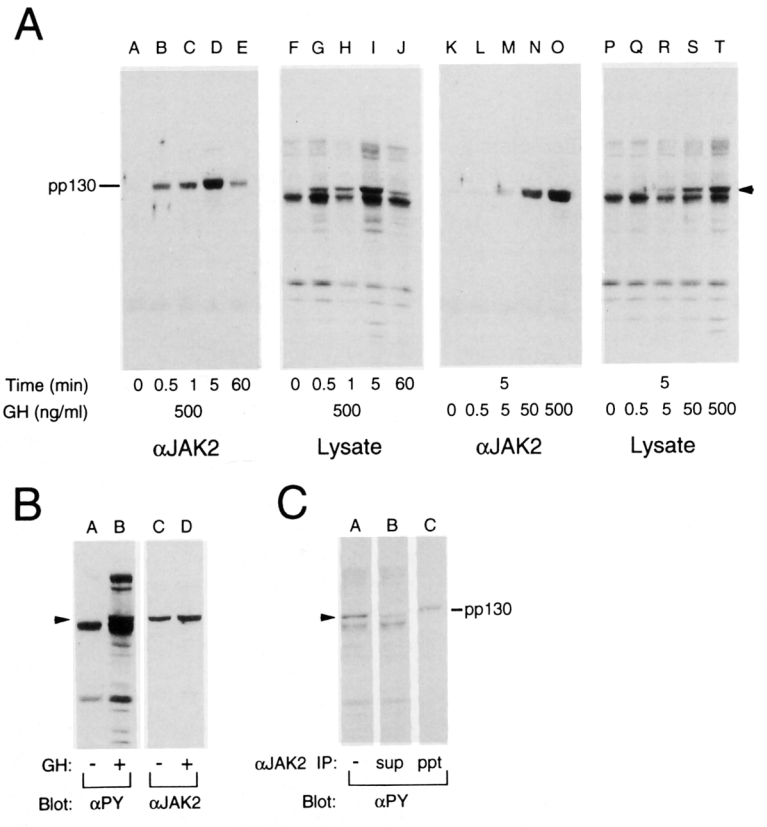

Figure 1. GH Promotes Tyrosyl Phosphorylation of JAK2

(A) 3T3-F442A fibroblasts were incubated with the indicated concentrations of hGH for the times shown. Whole-cell lysates were immunoprecipitated with αJAK2 (1:500 dilution). Immunoprecipitated proteins (lanes A–E and K–O) and unfractionated lysates (lanes F–J and P–T) were subjected to Western blot analysis using αPY-4G10. The decreased intensity of all the bands in lane H in comparison with the bands in the other lanes (including the 0 time point) suggests that lane H contains proteins from fewer cells than the other lanes.

(B) Whole-cell lysates of 3T3-F442A fibroblasts incubated without (lanes A and C) or with (lanes B and D) 500 ng/ml hGH for 5 min were subjected to Western blot analysis with either αPY-4G10 (lanes A and B) or αJAK2 (lanes C and D).

(C) Cell lysates from 3T3-F442A fibroblasts stimulated with 500 ng/ml hGH for 5 min were immunoprecipitated with αJAK2 (1:1000) and Western blotted with αPY-4G10. Lane A, total lysate; lane B, supernatant following immunoprecipitation with αJAK2; lane C, αJAK2 immunoprecipitate. All lanes represent equivalent amounts of cell lysate. When assessed by densitometry, the amount of pp121 removed by the αJAK2 treatment is found to be equal (within 5%) to the amount of tyrosyl-phosphorylated protein appearing in the αJAK2 immunoprecipitate. The migration of pp121 in whole-cell lysates is denoted by the arrow. The migration of pp130 is denoted.

cipitated using antiserum to JAK2 (αJAK2) and analyzed by anti-phosphotyrosine antibody (αPY) immunoblot (Figure 1A). GH-dependent tyrosyl phosphorylation of a protein with an M_r (~130,000) appropriate for JAK2 (Silvennoinen et al., 1993) was clearly evident at times as early as 30 s and at physiological concentrations of GH as low as 5.0 ng/ml (230 pM). Phosphorylation was transient, being greatly diminished by 60 min after addition of GH. The appearance of the 130 kd phosphoprotein in αPY immunoblots of αJAK2 immunoprecipitates (Figure 1A, lanes A–E and K–O) corresponds in time course and GH dose response with the appearance in whole-cell lysates of a tyrosyl-phosphorylated protein, indicated by the arrow in Figure 1A (lanes F–J and P–T) and designated pp121 in previous work (Campbell et al., 1993; Wang et al., 1993). The identity of these two proteins is suggested by the comigration in cell lysates of tyrosyl phosphorylated pp121 and JAK2 (Figure 1B) and the depletion of tyrosyl-phosphorylated pp121 from cell lysates following immunoprecipitation with αJAK2 (Figure 1C).

The 130 kd phosphoprotein was precipitated specifically by αJAK2 (Figures 2A and 2B, lanes B). Nonimmune serum (Figure 2A, lane A), an unrelated immune serum (αGLUT-1; Figure 2A, lane C), and αJAK2 preadsorbed with the peptide used to make the antibody (Figure 2B, lane D) failed to immunoprecipitate pp130. Preadsorption of αJAK2 with the analogous peptide from murine JAK1 (Silvennoinen et al., 1993) did not interfere with precipitation of the 130 kd phosphoprotein by αJAK2 (Figure 2B, lane F). In contrast with these results using αJAK2, immunoprecipitation of 3T3-F442A and IM-9 cell lysates, respectively, with antibodies specific for JAK1 (αJAK1) and Tyk2 (αTyk2) revealed little (αJAK1) or no (αTyk2) GH-dependent tyrosyl phosphorylation of a ~130 kd protein, despite the presence of these kinases in the respective cell types (data not shown).

Tyrosyl phosphorylation of the 130 kd protein precipitated from 3T3-F442A cells by αJAK2 was increased specifically by GH. Phosphorylation was not increased by platelet-derived growth factor, epidermal growth factor, or insulin-like growth factor 1 (Figure 2A, lanes E–G, respectively). These growth factors stimulate tyrosine kinase activity intrinsic to their receptors (Ullrich and Schlessinger, 1990) and promote tyrosyl phosphorylation of multiple proteins in 3T3-F442A fibroblasts (Campbell et al., 1993). The inability to stimulate JAK2 tyrosyl phosphorylation is consistent with the previously reported inability of these growth factors to stimulate tyrosyl phosphorylation of pp121 in whole-cell lysates (Campbell et al., 1993).

JAK2 Associates with the GH Receptor

To determine whether JAK2 forms a complex with GHR, GH–GHR complexes and associated proteins were immunoprecipitated from solubilized, GH-treated 3T3-F442A fibroblasts using antibody to GH (αGH). The presence of JAK2 in αGH immunoprecipitates was assessed either by

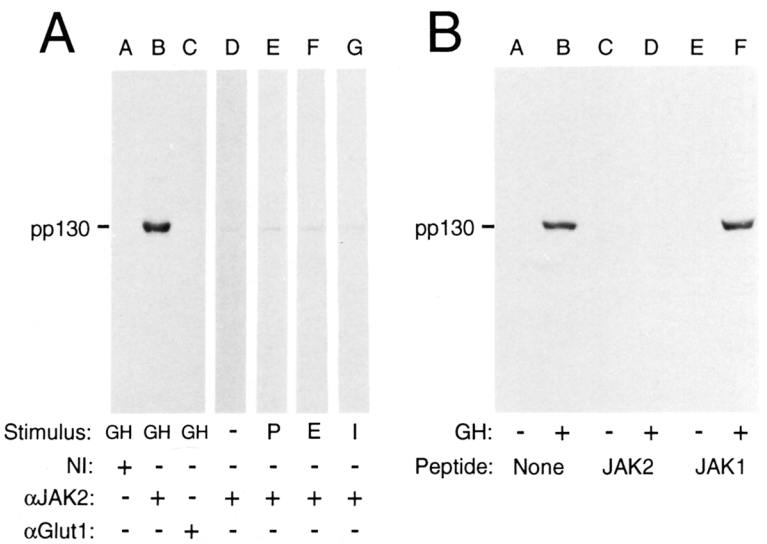

Figure 2. Tyrosyl Phosphorylation of JAK2 Is Specific to GH Stimulation

(A) Cell lysates from unstimulated 3T3-F442A fibroblasts (lane D) and from cells stimulated for 5 min with 500 ng/ml hGH (lanes A–C), 25 ng/ml platelet-derived growth factor (lane E), 125 ng/ml epidermal growth factor (lane F), or 200 ng/ml insulin-like growth factor 1 (lane G) were subjected to immunoprecipitation with αJAK2 (1:1000; lanes B and D–G), nonimmune serum (1:1000; lane A), or an irrelevant antiserum (αGLUT-1, 1:1000; lane C) (Tai et al., 1990), as described in Figure 1. The immunoprecipitates were analyzed by Western blot analysis with αPY-4G10.

(B) Cell lysates from unstimulated 3T3-F442A fibroblasts (lanes A, C, and E) and from cells stimulated for 5 min with 500 ng/ml hGH (lanes B, D, and F) were subjected to immunoprecipitation with αJAK2 (1:1000; lanes A and B), αJAK2 (1:1000) that had been preincubated for 1 hr at 0°C with the peptide (30 μg/ml) used as antigen to make αJAK2 (lanes C and D; Silvennoinen et al., 1993) or a peptide analogous to amino acids 785–804 of JAK1 (lanes E and F; Silvennoinen et al., 1993).

immunoblotting with αJAK2 or by immunoprecipitating with αJAK2 and immunoblotting with αPY. When material precipitated using αGH was analyzed, αJAK2 was found to immunoblot a 130 kd protein (Figure 3, lane B) and to immunoprecipitate a tyrosyl-phosphorylated 130 kd protein (Figure 3, lane L) that comigrates with a protein recognized by αJAK2 (Figure 3, lane M), indicating that JAK2 associates with GH–GHR complexes. When instead of αGH, the initial immunoprecipitation was performed with antibody to either the cytoplasmic (Figure 3, lanes C and D) or extracellular (data not shown) domains of GHR (αGHR), αJAK2 recognized a 130 kd protein only when cells had been incubated with GH. Consistent with the presence of JAK2 in the αGHR precipitate because of its association with GH-bound GHR, no signal was detected in αJAK2 immunoblots of αGH immunoprecipitates when cells had not been incubated with GH (Figure 3, lane A) nor when immunoprecipitation was performed using an unrelated immune serum (αGLUT-1) (data not shown). These results provide evidence that GH binding to its receptor is necessary for the formation of a complex between GHR and JAK2.

In addition to the 130 kd phosphoprotein believed to be JAK2, a diffusely migrating phosphoprotein of ~120 kd identified by αPY immunoblot was precipitated by αGH (Figure 3, lanes F and J), αGHR (Figure 3, lane H), and to a lesser extent αJAK2 (Figure 3, lane K). Consistent with this diffuse band being GHR, its size corresponds to that previously reported for GHR in these cells (Schwartz and Carter-Su, 1988; Stred et al., 1992), and it comigrates with a similarly diffuse ~120 kd band identified by αGHR in Western blots of αGH immunoprecipitates (Figure 3,

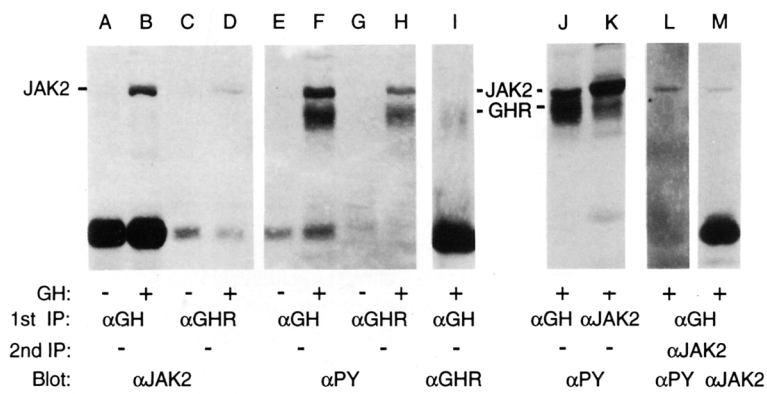

Figure 3. GH Promotes Complex Formation between GHR and JAK2 and Promotes Tyrosyl Phosphorylation of GHR and JAK2

3T3-F442A fibroblasts were incubated with vehicle (lanes A, C, E, and G) or 500 ng/ml hGH (lanes B, D, F, and H–M) for 5 min. Solubilized proteins were immunoprecipitated using αGH (1:4000; lanes A, B, E, F, I, J, L, and M), αGHR-C1 (~0.1 μg/ml; lanes C, D, G, and H), or αJAK2 (1:1000; lane K). The precipitated proteins were subjected to Western blotting using αJAK2 (lanes A–D), αPY-4G10 (lanes E–H, J, and K) or αGHR-C1 (~0.1 μg/ml; lane I). For the samples for lanes L and M, the precipitate was washed and boiled in buffer containing SDS, β-mercaptoethanol, and DTT, as described in Experimental Procedures. Samples were diluted 10-fold with lysis buffer, immunoprecipitated with αJAK2 (1:1000), and Western blotted using αPY-4G10 (lane L) or αJAK2 (lane M). Lanes A–H are from one experiment, lane I from a second, lanes J and K from a third, and lanes L and M from a fourth. The migrations of JAK2 and GHR are indicated.

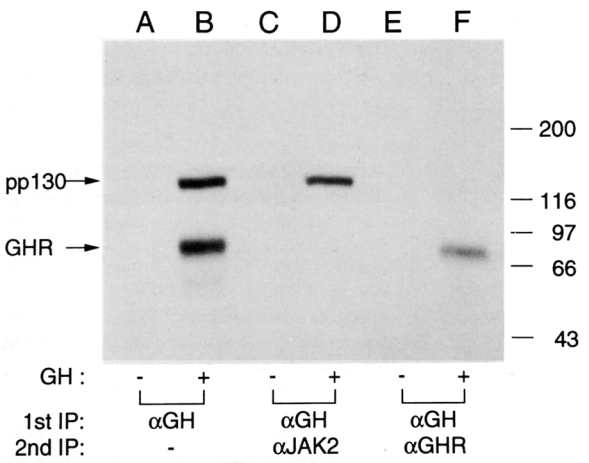

Figure 4. JAK2 Is Associated with GHR and Is Phosphorylated In Vitro

CHO4 cells were incubated at 25°C in the absence (lanes A, C, and E) or presence (lanes B, D, and F) of 30 ng/ml hGH for 60 min. Cellular proteins were solubilized, immunoprecipitated using αGH, and incubated with [γ-^{32}P]ATP. Immune complexes and associated proteins were treated with SDS, β-mercaptoethanol, and DTT. The dissociated proteins were diluted. Samples were then removed for analysis by SDS–PAGE (150 μl; lanes A and B) or reimmunoprecipitated (550 μl) with αJAK2 (lanes C and D) or αGHR (αGHBP-poly; lanes E and F) as described in Experimental Procedures. ^{32}P-labeled proteins were resolved by SDS–PAGE and visualized by autoradiography. The molecular weight (× 10^{-3}) of protein standards and the migration of pp130 and GHR are indicated.

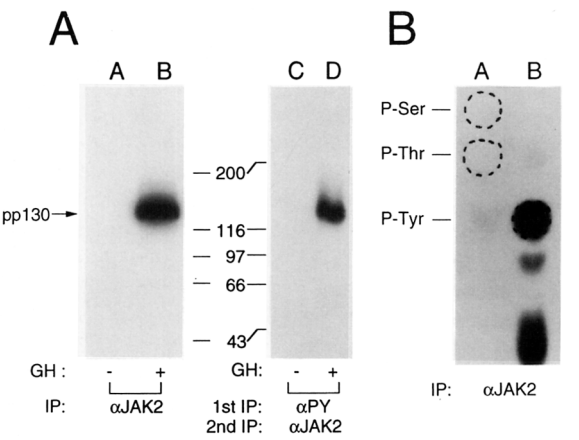

Figure 5. Purified JAK2 Possesses Tyrosine Kinase Activity

(A) 3T3-F442A cells were incubated at 25°C in the absence (lanes A and C) or presence (lanes B and D) of 30 ng/ml hGH for 1 hr. Solubilized proteins were immunoprecipitated using αJAK2 (lanes A and B) or sequentially immunoprecipitated using first αPY and then αJAK2 (lanes C and D) and incubated with [γ-^{32}P]ATP as described in Experimental Procedures. The molecular weight (× 10^{-3}) of protein standards and migration of pp130 are indicated.

(B) pp130 was excised from the gel visualized in lane B of (A) and subjected to limited acid hydrolysis at 109°C for 1.25 hr. Following partial purification on Dowex-50, fractions containing O-phosphoserine and O-phosphothreonine (lane A) or O-phosphotyrosine (lane B) were resolved by thin layer electrophoresis (pH 3.5) as previously described (Carter-Su et al., 1989; Stred et al., 1990; Wang et al., 1992). The migrations of O-phosphoserine (P-Ser), O-phosphothreonine (P-Thr), and O-phosphotyrosine (P-Tyr) standards are indicated by the dashed circles.

lane I). The finding that tyrosyl residues are phosphorylated in the diffuse 120 kd protein present in αGHR immunoprecipitates only when the cells have been incubated with GH (Figure 3, lanes G and H) offers evidence that tyrosyl phosphorylation of GHR, like tyrosyl phosphorylation of JAK2, is GH dependent. Additional evidence that both JAK2 and GHR are tyrosyl phosphorylated in response to GH is provided by the finding that in a transfected Chinese hamster ovary cell line (CHO4) that expresses a smaller (84 kd) GHR (Emtner et al., 1990; Wang et al., 1993), tyrosyl phosphorylation of a 130 kd protein in αGH, αGHR, and αJAK2 immunoprecipitates and a diffusely migrating 84 kd protein in αGH and αGHR immunoprecipitates is GH dependent (data not shown).

Stimulation by GH of JAK2 Kinase Activity

Previous studies have established that when αGH precipitates are prepared from GH-treated CHO4 cells, the addition of ATP results in the tyrosyl phosphorylation of both a 130 kd and a 84 kd protein (Wang et al., 1993). To determine whether the 130 kd and 84 kd proteins phosphorylated in this in vitro kinase assay are JAK2 and GHR, respectively, GH–GHR complexes and associated proteins were precipitated from GH-treated CHO4 cells using αGH, incubated with [γ-^{32}P]ATP, dissociated by boiling in buffer containing SDS, β-mercaptoethanol, and dithiothreitol (DTT), and reprecipitated using either αJAK2 or αGHR. The ability of αJAK2 to precipitate a 130 kd ^{32}P-labeled protein appropriate for JAK2 (Figure 4, lane D), as well as the ability of αGHR to precipitate an 84 kd ^{32}P-labeled protein appropriate for GHR (Figure 4, lane F), indicates that both JAK2 and GHR incorporate ^{32}P in the in vitro kinase assay.

To verify that JAK2 functions as a GH-dependent tyrosine kinase, JAK2 was purified from GH-treated and control 3T3-F442A cells either by direct immunoprecipitation with αJAK2 (Figure 5A, lanes A and B) or, to permit a higher degree of purification, by sequential immunoprecipitation using αPY followed by αJAK2 (Figure 5A, lanes C and D). When the αJAK2 immune complexes were incubated with [γ-^{32}P]ATP, ^{32}P-labeled proteins migrating with a M_r (130,000) appropriate for JAK2 were detected only when the cells had been incubated with GH (Figure 5A, lanes B and D), indicating an exquisite sensitivity of JAK2 to activation by GH. To verify that JAK2 incorporates phosphate into tyrosyl residues, phosphoamino acid analysis was performed on the ^{32}P-labeled 130 kd protein isolated from GH-treated 3T3-F442A cells (Figure 5A, lane B). ^{32}P was incorporated almost exclusively into tyrosyl residues (Figure 5B), consistent with JAK2 being a GH-sensitive tyrosine kinase. However, the incorporation of a small amount of ^{32}P (under 1%) into threonine residues in the αJAK2 immunoprecipitate leaves open the possibility that JAK2 is a mixed function threonine/serine/tyrosine kinase.

Discussion

Identification of JAK2 As a Signaling Molecule for GHR

The identification of JAK2 as a GH-dependent, GHR-associated tyrosine kinase has important implications for signal transduction by both GHR and JAK2. With regard to GHR, JAK2 is identified as a signaling molecule that interacts with GHR and is activated in response to GH binding. Its sensitivity to GH and rapid onset following GH addition make tyrosyl phosphorylation of JAK2 among the most sensitive and rapid responses known for GH, raising the possibility that activation of JAK2 is the initiating step for GH signal transduction. Tyrosine kinases have been shown to elicit responses similar to those attributable to GH, including metabolic responses (e.g., insulin receptor) and differentiation (e.g., nerve growth factor receptor) (reviewed by Davidson, 1987; Isaksson et al., 1987; Levi-Montalcini, 1987; Kaplan et al., 1991). Therefore, it seems likely that JAK2 plays a vital role in eliciting at least some of the known responses to GH. Consistent with this, no biological functions, other than binding of GH, have been reported for GHR expressed in cells that have low levels of GHR-associated tyrosine kinase activity (e.g., COS-7 and mouse L cells; Leung et al., 1987; Wang et al., 1992, and unpublished data). In contrast, a variety of biological functions (e.g., insulin synthesis in RIN5-AH cells and protein synthesis, microtubule-associated protein kinase activity, c-*fos* gene expression, and lipid synthesis in Chinese hamster ovary cells) can be activated by GH binding when the cloned liver GHR is expressed in cells that have reasonably high levels of GHR-associated kinase activity (Billestrup et al., 1990; Emtner et al., 1990; Moller et al., 1992; Wang et al., 1992; Moller, 1992; Wang et al., submitted). Furthermore, in 3T3-F442A cells, multiple proteins exhibit GH-dependent increases in tyrosyl phosphorylation. Consistent with activation of JAK2 being required for these phosphorylations, tyrosyl phosphorylation of JAK2/pp121 is simultaneous with or precedes tyrosyl phosphorylation of all the proteins exhibiting GH-dependent tyrosyl phosphorylation, at all GH concentrations tested (this work and Campbell et al., 1993).

Presumably, JAK2 serves as a signaling molecule for GHR by phosphorylating other proteins. Thus far, only two proteins have been identified as likely substrates of JAK2: JAK2 itself and GHR. Studies using truncated GHR indicate that in the cytoplasmic domain of the GHR, at least 1 of the 4 tyrosyl residues most proximal to the membrane is phosphorylated in response to GH (Wang et al., submitted). Studies are underway to identify which of the 4 tyrosines are phosphorylated by JAK2, as well as to identify tyrosines in the C-terminal portion of GHR that might also be phosphorylated. It is important to determine the identity and number of tyrosines phosphorylated in JAK2 and GHR, because these sites are likely to be binding sites for SH2-containing proteins (e.g., phospholipase C-γ, p85 phosphatidylinositol-3 kinase, and GTPase-activating protein; Koch et al., 1991) in intracellular signaling pathways. Signaling pathways involving SH2-containing proteins that bind to phosphorylated JAK2 would be expected to be shared by all ligands that activate JAK2, whereas SH2-containing proteins that bind to phosphorylated tyrosyl residues in GHR could provide specificity to a signaling mechanism that utilizes a kinase (i.e., JAK2) with the apparent capacity to service more than one receptor (see below).

JAK2 has also been shown to be activated following the binding of EPO to its receptor (Witthuhn et al., 1993). Other data indicate that IL-3, GM-CSF, G-CSF, IFN-γ, and prolactin also activate JAK2 (Silvennoinen et al., 1993; Witthuhn et al., 1993; O. S. et al., unpublished data; G. S. C. et al., unpublished data). Thus, it appears that JAK2 serves as a kinase for multiple members of the cytokine/hematopoietin receptor family. Since each ligand elicits a separate constellation of responses, kinase activation alone cannot account for specificity. As mentioned above, a set of responses dependent upon phosphorylation of the receptor could provide this specificity. Additionally, specificity could be obtained by interaction between multiple signaling pathways or by the expression of only one receptor type in a particular cell type. This latter mechanism is suggested by the ability of GH, G-CSF, and EPO to stimulate proliferation of IL-3-dependent cells transfected with the cDNA for the appropriate receptor (Fukunaga et al., 1991; Ishizaka-Ikeda et al., 1993; Yoshimura et al., 1990).

The commonality of JAK2 activation suggests that there will be shared pathways activated by the ligands that bind JAK2-coupled receptors. Of particular interest for gaining insight into regulation of gene transcription by GH is a pathway initiated by IFN-γ. In response to IFN-γ, the 91 kd protein of the ISGF-3 (IFN-stimulated gene factor 3) complex undergoes tyrosyl phosphorylation and then translocates to the nucleus, where it binds to DNA at the γ-activated site (Shuai et al., 1992). Identification of the 90 kd protein phosphorylated in response to GH (Campbell et al., 1993) as the 91 kd protein of the ISGF-3 complex or a family member would implicate one pathway by which GH might elicit some of its effects on gene transcription.

Activation of JAK2 by GH

The exact mechanism by which GH activates JAK2 is not yet known. Earlier studies using an exogenous substrate (poly Glu, Tyr) established that more tyrosine kinase activity is present in a complex with GHR when GHR is prepared from GH-treated cells than from control cells (Stred et al., 1992). The present study suggests that this GH-induced increase in kinase activity results from both an increase in affinity of GHR for JAK2 and an increase in JAK2 activity. JAK2 appears to bind directly to GHR, since only two proteins, migrating with sizes appropriate for JAK2 and GHR, are visualized when highly purified kinase-active GH–GHR complexes are isolated from GH-treated ^{35}S-labeled 3T3-F442A fibroblasts by sequential immunoprecipitation using αPY and then either αGHR or αGH (Stred et al., 1992). The mechanism by which GH pro-

motes association of JAK2 with GHR and JAK2 activation is likely to require dimerization of GHR, since GH-induced tyrosyl phosphorylation of cellular proteins appears to require dimerization of GHR (Silva et al., 1993). An important role for receptor dimerization in signaling via JAK2 is further suggested by work relating JAK2 activation to EPO receptor dimerization discussed by Witthuhn et al. (1993).

On the basis of these findings, we hypothesize that the binding of GH by GHR results in the formation of a ligand-bound GHR dimer capable of binding JAK2. Recruitment of JAK2 leads to the formation of a GH–GHR–JAK2 complex, stimulation of JAK2 tyrosine kinase activity, and tyrosyl phosphorylation of JAK2, GHR, and presumably other proteins. Whether activated JAK2 is present only in a complex with GHR or can dissociate from GHR and phosphorylate proteins that are physically distant from GHR is currently being investigated. Also under investigation is the possibility that GHR can form complexes with kinases other than, or in addition to, JAK2. Obvious candidate kinases include other members of the JAK family. In 3T3-F442A and IM-9 cells, respectively, JAK1 and Tyk2 do not appear to associate with GHR to the same extent as JAK2. However, they or other as yet unidentified JAK kinases may do so in other cell types or under different physiological conditions.

It will be intriguing to determine whether the interactions between JAK2 and the receptors for IL-3, EPO, GM-CSF, G-CSF, prolactin, and IFN-γ, like the interaction between JAK2 and GHR, is ligand dependent. In the accompanying paper (Witthuhn et al., 1993), JAK2 from either EPO-stimulated or unstimulated cells is reported to bind to a fusion protein composed of glutathione S-transferase and the cytoplasmic domain of the EPO receptor. This suggests that JAK2 may be constitutively associated with some of the receptors capable of activating JAK2.

In summary, the experiments presented in the present paper, in combination with the similar findings for the EPO receptor presented in the accompanying paper and other work with the receptors for IL-3, GM-CSF, G-CSF, prolactin, and IFN-γ (Silvennoinen et al., 1993; Witthuhn et al., 1993; O. S. et al., unpublished data; G. S. C. et al., unpublished data), suggest that the activation of JAK2 kinase activity by GH and EPO by a mechanism involving a JAK2–receptor complex may be a prototype for signaling by many members of the cytokine/hematopoietin family receptors. The finding that GHR shares an important and early signaling molecule with other members of the cytokine/hematopoietin receptor family suggests that GH, IL-3, EPO, prolactin, GM-CSF, G-CSF and IFN-γ are likely to share some signaling pathways. However, specificity could still be achieved, since phosphorylation of each receptor offers signaling capabilities unique to each ligand. The variable expression of individual receptors, the potential presence of only a subset of all possible signaling pathways in different cell types, and regulation of the signaling molecules in these pathways by other stimuli permits an additional level of specificity. This finding is likely to lead to the identification of new actions for GH as well as for these other cytokines.

Experimental Procedures

Materials

Stocks of 3T3-F442A and CHO4 cells were kind gifts of H. Green (Harvard University, Cambridge, MA) and G. Norstedt (Karolinska Institute, Novum, Sweden), respectively. Recombinant human GH (hGH) was provided by Eli Lilly. Platelet-derived growth factor (recombinant human BB) and recombinant epidermal growth factor came from Collaborative Research. Recombinant insulin-like growth factor 1 was a gift of Kabi/Pharmacia. Triton X-100 (Surfact-Amps X-100) came from Pierce Chemical Company, aprotinin and leupeptin from Boehringer Mannheim, recombinant protein A–agarose from Repligen, [γ-^{32}P]ATP (6000 Ci/mmol) from New England Nuclear Corporation, and the enhanced chemiluminescence detection system from Amersham Corporation.

Antibodies

αGH (NIDDK-anti-hGH-IC3, lot C11981) came from the National Institute of Diabetes and Digestive and Kidney Diseases/National Hormone and Pituitary Program, University of Maryland School of Medicine (Baltimore). αPY-Shafer was a gift of Dr. J. A. Shafer (Merck, Sharp, and Dohme Research Laboratory, West Point, PA; Pang et al., 1985), and αPY-4G10 was purchased from UBI. αJAK2 was prepared in rabbits against a synthetic peptide corresponding to the hinge region between domains 1 and 2 of murine JAK2 (amino acids 758–776; Silvennoinen et al., 1993). αJAK1 was prepared against a synthetic peptide to a corresponding region in murine JAK1 (amino acids 785–804; Silvennoinen et al., 1993). One αGHR (αGHR-C1) was prepared in rabbits against a fusion protein composed of glutathione S-transferase fused to the cytoplasmic domain of the cloned mouse liver GHR and affinity purified using immobilized GHR cytoplasmic domain (L. S. A. et al., unpublished data). A second αGHR (αGHBP-poly), kindly provided by Dr. W. R. Baumbach (American Cyanamid, Princeton, NJ), was produced in rabbits using recombinant rat GH-binding protein produced in Escherichia coli (Sadeghi et al., 1990). αTyk2 was a gift of Dr. J. J. Krolewski (Columbia University, New York). αGLUT-1 was prepared in rabbits using band 4.5 purified from human erythrocytes. It recognizes both human and rodent GLUT-1 (Tai et al., 1990).

Immunoprecipitation and Western Blotting

Cells were grown to confluence and deprived of serum overnight as described previously (Wang et al., 1993). Cells were incubated for the indicated times with hormone or growth factor as indicated at 37°C in 95% air, 5% CO_2, rinsed with three changes of ice-cold 10 mM sodium phosphate (pH 7.4), 137 mM NaCl, 1 mM Na_3VO_4, and scraped in lysis buffer (50 mM Tris [pH 7.5], 0.1% Triton X-100, 137 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) on ice. Cell lysates were centrifuged at 12,000 × g for 10 min, and the resulting supernatants were incubated on ice for 90 min with the indicated antibody. Immune complexes were collected on protein A–agarose during a 30–60 min incubation at 8°C, washed three times with wash buffer (50 mM Tris [pH 7.5], 0.1% Triton X-100, 137 mM NaCl, 2 mM EGTA) and boiled for 5 min in a mixture (80:20) of lysis buffer and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer (250 mM Tris [pH 6.8], 10% SDS, 10% β-mercaptoethanol, 40% glycerol). Unfractionated lysates were brought to the same final concentrations of Tris, SDS, β-mercaptoethanol, and glycerol and boiled for 5 min. The immunoprecipitates and lysates were subjected to SDS–PAGE followed by Western blot analysis with the indicated antibody (1:5000 dilution unless noted otherwise) using the enhanced chemiluminescence detection system (Campbell et al., 1993). In some experiments, the proteins were dissociated from the immune complexes and then reimmunoprecipitated before analysis by Western blot.

Dissociation and Reimmunoprecipitation of Immune Complexes

The immune complexes from the initial immunoprecipitation were washed once with 50 mM Tris, 137 mM NaCl (pH 7.5), brought to a final concentration of 0.75% SDS, 2% β-mercaptoethanol, 100 mM DTT, 100 μg/ml aprotinin, and 100 μg/ml leupeptin by addition of an equal volume of a 2× concentrated stock, and then boiled for 5 min.

The eluted proteins were diluted 10-fold with lysis buffer. A portion was removed, mixed (80:20) with SDS–PAGE sample buffer, and boiled for 5 min. The remaining sample was incubated with the second antiserum on ice for 60–90 min and with protein A–agarose at 8°C for 1 hr. The immune complexes were washed three times with lysis buffer and boiled for 5 min in a mixture (80:20) of wash buffer and SDS–PAGE sample buffer.

Immunoprecipitation for Kinase Assays

Serum-deprived cells were incubated at 25°C in the absence or presence of 30 ng/ml hGH for 60 min. The relatively long incubation period, low GH concentration, and low temperature were used to maximize the in vitro incorporation of ^{32}P into pp130 and GHR during the kinase assay. Cells were washed with phosphate-buffered saline, solubilized in 25 mM HEPES, 2 mM Na_3VO_4, 0.1% Triton X-100, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin (pH 7.4) (HVT), and centrifuged at 200,000 × g for 1 hr at 4°C. Soluble proteins were incubated on ice for 1 hr with either αGH (1:10,000 dilution), αPY-Shafer (15 μg per plate of cells), or αJAK2 (1:1,500 dilution) (Carter-Su et al., 1989). Protein A–agarose was added for an additional 1 hr at 8°C. Immune complexes were washed three times with 50 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 0.5 mM DTT (pH 7.6) (NHT) and then once with 50 mM HEPES, 100 mM NaCl, 6.25 mM $MnCl_2$, 0.1% Triton X-100, 0.5 mM DTT (pH 7.6) (HNMT).

Sequential Immunoprecipitation with αPY and αJAK2

Proteins immobilized on αPY-protein A–agarose complexes were transferred to a small plastic column and equilibrated for 5 min with 10 mM p-nitrophenyl phosphate, 20 μg/ml aprotinin, 20 μg/ml leupeptin in HNMT (eluting buffer). Phosphoproteins were then eluted with 180 μl of eluting buffer. αJAK2 (1:200 dilution) was added, and the mixture was incubated on ice for 1 hr. Protein A–agarose and 0.7 ml of HNMT containing 20 μg/ml aprotinin, 20 μg/ml leupeptin (phosphorylation buffer) was added, and incubation continued at 8°C for 1 hr. Immune complexes were washed three times with NHT and once with phosphorylation buffer.

In Vitro Kinase Assay and Phosphoamino Acid Analysis

Proteins immobilized on αJAK2 or αGH were mixed with 95 μl of phosphorylation buffer. [γ-^{32}P]ATP was then added to yield a final concentration of 10 μM ATP and 5 mM $MnCl_2$. After 10 min at 30°C, the reaction was stopped with the addition of 10 mM EDTA in NHT. The immune complexes were washed three times with NHT and once with phosphorylation buffer. ^{32}P-labeled proteins were either subjected to a second immunoprecipitation or boiled for 5 min in SDS–PAGE sample buffer, resolved by SDS–PAGE, and visualized by autoradiography. The phosphoamino acid content of phosphorylated proteins was determined by limited acid hydrolysis using a modification of the procedure of Hunter and Sefton (1980) as described previously (Carter-Su et al., 1989; Stred et al., 1990; Wang et al., 1992).

SDS–PAGE and Densitometry

Proteins were separated by SDS–PAGE on 3%–10% gradient gels (30:0.05 acrylamide:bisacrylamide) as described previously (Carter-Su et al., 1989). Densitometry was performed using a Bio-Med Instruments laser scanning densitometer attached to an Apple IIE computer (Bio-Med Instruments Videophoresis II data analysis computer program).

Acknowledgments

L. S. A. and G. S. C. contributed equally to the work and should both be considered first authors. This work was supported in part by research grants from the National Institutes of Health to C. C.-S. (RO1-DK34171), J. N. I. (RO1-DK42932 and P30 CA21765), and C. Cepko (RO1-EY08064); a grant from the American Lebanese Syrian Associated Charities to J. N. I.; National Research Service Award postdoctoral fellowships from the National Institutes of Health to L. S. A. (1F32-DK08737) and G. S. C. (1F32-GM14099); and postdoctoral fellowships to X. Y. from the Helen Hay Whitney Foundation and the Medical Foundation/Charles King Trust. We thank Drs. R. Jove, J. Schwartz, M. Uhler, and C. Cepko for helpful comments.

Received May 6, 1993; revised June 1, 1993.

References

Bazan, J. F. (1990). Structural design and molecular evolution of a cytokine receptor superfamily. Proc. Natl. Acad. Sci. USA *87*, 6934–6938.

Billestrup, N., Moldrup, A., Serup, P., Mathews, L. S., Norstedt, G., and Nielsen, J. (1990). Introduction of exogenous growth hormone receptors augments growth hormone-responsive insulin biosynthesis in rat insulinoma cells. Proc. Natl. Acad. Sci. USA *87*, 7210–7214.

Campbell, G. S., Christian, L. J., and Carter-Su, C. (1993). Evidence for involvement of the growth hormone receptor–associated tyrosine kinase in actions of growth hormone. J. Biol. Chem. *268*, 7427–7434.

Carter-Su, C., Stubbart, J. R., Wang, X., Stred, S. E., Argetsinger, L. S., and Shafer, J. A. (1989). Phosphorylation of highly purified growth hormone receptors by a growth hormone receptor-associated tyrosine kinase. J. Biol. Chem. *264*, 18654–18661.

Cheek, D. B., and Hill, D. E. (1974). Effect of growth hormone on cell and somatic growth. In Handbook of Physiology, Volume 4, E. Knobil and W. H. Sawyer, eds. (Washington DC: American Physiological Society), pp. 159–185.

Davidson, M. B. (1987). Effect of growth hormone on carbohydrate and lipid metabolism. Endocrinol. Rev. *8*, 115–131.

Emtner, M., Mathews, L. S., and Norstedt, G. (1990). Growth hormone (GH) stimulates protein synthesis in cells transfected with GH receptor complementary DNA. Mol. Endocrinol. *4*, 2014–2020.

Firmbach-Kraft, I., Byers, M., Shows, T., Dalla-Favera, R., and Krolewski, J. J. (1990). Tyk2, prototype of a novel class of non-receptor tyrosine kinase genes. Oncogene *5*, 1329–1336.

Fukunaga, R., Ishizaka-Ikeda, R., Pan, C.-X., Seto, Y., and Nagata, S. (1991). Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. *10*, 2855–2865.

Harpur, A. G., Andres, A.-C., Ziemiecki, A., Aston, R. R., and Wilks, A. F. (1992). JAK2, a third member of the JAK family of protein tyrosine kinases. Oncogene *7*, 1347–1353.

Hunter, T., and Sefton, B. M. (1980). Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA *77*, 1311–1315.

Isaksson, O. G. P., Lindahl, A., and Isgaard, J. (1987). Mechanism of the stimulatory effect of growth hormone on longitudinal bone growth. Endocrinol. Rev. *8*, 426–438.

Ishizaka-Ikeda, E., Fukunaga, R., Wood, W. I., Goeddel, D. V., and Nagata, S. (1993). Signal transduction mediated by growth hormone receptor and its chimeric molecules with the granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA *90*, 123–127.

Kaplan, D. R., Hempstead, B., Martin-Zanca, D., Chao, M., and Parada, L. F. (1991). The *trk* proto-oncogene product: a signal transducing receptor for nerve growth factor. Science *252*, 554–558.

Koch, A. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science *252*, 668–674.

Leung, D. W., Spencer, S. A., Cachianes, G., Hammonds, R. G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J., and Wood, W. I. (1987). Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature *330*, 537–543.

Levi-Montalcini, R. (1987). The nerve growth factor 35 years later. Science *237*, 1154–1162.

Moller, C. (1992). Growth hormone regulation of lipid metabolism in cells transfected with rat GH receptor cDNA. In Aspects of the Mechanism of Growth Hormone Action. PhD thesis, Karolinska Institute, NOVUM, Huddinge, Sweden, Section 4 pp. 1–9.

Moller, C., Hansson, A., Enberg, B., Lobie, P. E., and Norstedt, G. (1992). Growth hormone induction of tyrosine phosphorylation and activation of mitogen activated protein kinases in cells transfected with rat GH receptor cDNA. J. Biol. Chem. *267*, 23403–23408.

Pang, D. T., Sharma, B. R., and Shafer, J. A. (1985). Purification of

the catalytically active phosphorylated form of insulin receptor kinase by affinity chromatography with 0-phosphotyrosine-binding antibodies. Arch. Biochem. Biophys. *242*, 176–186.

Sadeghi, H., Wang, B. S., Lumanglas, A. L., Logan, J. S., and Baumbach, W. R. (1990). Identification of the origin of the growth hormone-binding protein in rat serum. Mol. Endocrinol. *4*, 1799–1805.

Schwartz, J., and Carter-Su, C. (1988). Effects of growth hormone on glucose metabolism and glucose transport in 3T3-F442A cells: dependence on cell differentiation. Endocrinology *122*, 2247–2256.

Shuai, K., Schindler, C., Prezioso, V. R., and Darnell, J. E., Jr. (1992). Activation of transcription by IFN-γ: tyrosine phosphorylation of a 91-kD DNA binding protein. Science *258*, 1808–1812.

Silva, C. M., Weber, M. J., and Thorner, M. O. (1993). Stimulation of tyrosine phosphorylation in human cells by activation of the growth hormone receptor. Endocrinology *132*, 101–108.

Silvennoinen, O., Witthuhn, B., Quelle, F. W., Cleveland, J. L., Yi, T., and Ihle, J. N. (1993). Structure of the JAK2 protein tyrosine kinase and its role in IL-3 signal transduction. Proc. Natl. Acad. Sci. USA, in press.

Stred, S. E., Stubbart, J. R., Argetsinger, L. S., Shafer, J. A., and Carter-Su, C. (1990). Demonstration of growth hormone (GH) receptor-associated tyrosine kinase activity in GH-responsive cell types. Endocrinology *127*, 2506–2516.

Stred, S. E., Stubbart, J. R., Argetsinger, L. S., Smith, W. C., Shafer, J. A., Talamantes, F., and Carter-Su, C. (1992). Stimulation by growth hormone (GH) of GH receptor-associated tyrosine kinase activity. Endocrinology *130*, 1626–1636.

Tai, P.-K. K., Liao, J.-F., Chen, E. H., Dietz, J. J., and Carter-Su, C. (1990). Differential regulation of two glucose transporters by chronic growth hormone treatment of cultured 3T3-F442A adipose cells. J. Biol. Chem. *265*, 21828–21834.

Ullrich, A., and Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. Cell *61*, 203–212.

Velazquez, L., Fellous, M., Stark, G. R., and Pellegrini, S. (1992). A protein tyrosine kinase in the interferon α/β signaling pathway. Cell *70*, 313–322.

Wang, X., Uhler, M., Billestrup, N., Norstedt, G., Talamantes, F., Nielsen, J. H., and Carter-Su, C. (1992). Evidence for association of the cloned liver growth hormone receptor with a tyrosine kinase. J. Biol. Chem. *267*, 17390–17396.

Wang, X., Moller, C., Norstedt, G., and Carter-Su, C. (1993). Growth hormone promoted tyrosyl phosphorylation of a 121-kDa growth hormone receptor-associated protein. J. Biol. Chem. *268*, 3573–3579.

Wilks, A. F., Harpur, A. G., Kurban, R. R., Ralph, S. J., Zurcher, G., and Ziemiecki, A. (1991). Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. Mol. Cell. Biol. *11*, 2057–2065.

Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle, J. N. (1993). JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell *74*, this issue.

Yoshimura, A., D'Andrea, A. D., and Lodish, H. F. (1990). Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. Proc. Natl. Acad. Sci. USA *87*, 4139–4143.