Participation of JAK and STAT Proteins in Growth Hormone-induced Signaling*

Yulong Han†, Douglas W. Leaman, Diane Watling§, Neil C. Rogers§, Bernd Groner†, Ian M. Kerr§, William I. Wood§, and George R. Stark‡**

*From the Department of Molecular Biology, Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio 44195, the Imperial Cancer Research Fund, Lincoln’s Inn Fields, London WC2A 3PX, United Kingdom, the Institute for Experimental Cancer Research, Tumor Biology Center, D79106 Freiburg, Federal Republic of Germany, and the Department of Molecular Biology, Genentech, Inc., South San Francisco, California 94080.

The binding of growth hormone leads to dimerization of its receptor, accompanied by phosphorylation and activation of intracellular tyrosine kinases (J AKs) and the latent cytoplasmic transcription factors STAT1, STAT3, and STAT5. Both JAK1 and JAK2 are phosphorylated in response to growth hormone in mouse 3T3 F442A and human HT1080 cells. The roles of JAKs in growth hormone signal transduction were examined by using mutant HT1080 cells missing either JAK1 or JAK2. JAK2 is absolutely required for growth hormone-dependent phosphorylation of the receptor, STAT1 and STAT3, JAK3, and the SH2-containing adaptor molecule Shc. In contrast, JAK1 is not required for any of the above functions. These data indicate that JAK2 is both necessary and sufficient for the growth hormone-dependent phosphorylation events required to couple the receptor both to STAT-dependent signaling pathways and to pathways involving Shc. Furthermore, STAT5 is activated by growth hormone in 3T3 F442A cells, but not in HT1080 cells, revealing that the set of STATs activated by growth hormone can vary, possibly contributing to the specificity of the growth hormone response in different cell types.

GH (growth hormone) regulates important physiological processes, such as fat metabolism and growth of the long bones (1). Its effects are mediated through activation of the GHR, a 134-kDa glycoprotein belonging to the cytokine/hematopoietin receptor superfamily (2–5). In response to GH, the GHR itself (7) and members of the STAT family of transcription factors (8) are phosphorylated rapidly (9–11). These events are accompanied by phosphorylation on tyrosine and activation of the receptor-associated tyrosine kinase JAK2 (12). Although JAK and STAT activations correlate with the GH-dependent proliferation of lymphoid cell lines (13), other signaling pathways also contribute to the full GH response. GH has been shown to activate mitogen-activated protein kinases and to promote phosphorylation on tyrosine of Shc and the association of Shc with Grb2 (14), implicating Ras-dependent modes of signaling that may or may not be linked to the JAK-STAT pathway.

JAKs and STATs are now known to transduce signals initiated by many growth factors and cytokines (6). Although many of these ligands activate overlapping sets of STATs, some, such as IFN-α and IL-4, activate unique family members (STAT2 and STAT6, respectively) known to be involved in other pathways (6). Furthermore, activated STAT monomers associate to form homo- and heterodimers, which can interact with other DNA-binding proteins to produce complex transcription factors with different sequence specificities (15), providing an additional level of regulation. Finally, it is clear that some of the extracellular signaling proteins that activate JAK-STAT pathways also trigger Ras-dependent pathways, which can activate the transcription of distinct sets of genes (16). At least three different family members (STAT1, STAT3, and STAT5) are activated by GH (9, 17–20). JAK2 is the only family member yet implicated in GH signaling (12).

We have used a genetic approach to isolate cell lines defective in IFN-α- or IFN-γ-dependent signaling (21–23). Mutant cells have been isolated in eight complementation groups, five of which lack individual JAK or STAT proteins (reviewed in Ref. 8). These cells have been extremely useful in characterizing the roles of individual components in IFN-dependent signaling and in evaluating the roles of these molecules in transducing signals initiated by other factors, such as IL-6 (24) and EGF (25). In this report, we have used some of the mutant cell lines to assess the roles of individual JAKs and STATs in GH-dependent signaling.

**MATERIALS AND METHODS**

Cell Lines, Ligands, and Antibodies—The parental cell lines 2TGH and 2C4 and the IFN-unresponsive mutant lines U1A (lacking Tyk2), U3A (lacking STAT1), and U6A (lacking STAT2) have been described (26–30). The mutant lines U4C (missing JAK1) and U2A (missing JAK2) were isolated as described by Watling et al. (30). The 3T3 preadipocyte fibroblast line F442A was kindly provided by Dr. H. Green (Harvard University). Recombinant human GH and IFN-γ were obtained from Genentech, Inc., and IFN-α (a mixture of lymphoblastoid subtypes) was obtained from Wellcome Research Laboratories. GH, IFN-α, and IFN-γ were used at final concentrations of 500 ng/ml, 1000 IU/ml, and 500 IU/ml, respectively.

The following antibodies were used: anti-JAK1 from A. Ziemiecki (University of Berne, Berne, Switzerland); anti-JAK2 and anti-STAT6 from J. Ihle (St. Jude’s, Memphis, TN); anti-STAT1 and anti-STAT2 from J. Darnell, J. F. (Rockefeller University, New York); anti-STAT3 from D. Levy (New York University); and anti-STAT5, raised against a synthetic peptide corresponding to amino acids 5–107 of sheep STAT5 (31). Also used were anti-phosphotyrosine monoclonal antibodies

---

**This work was supported by National Institutes of Health Postdoctoral Training Grant F32AI08956 (to D. W. L.) and Grant P01CA62220 (to G. R. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.**

**To whom correspondence should be addressed: Research Inst./NCI, Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-444-3900; Fax: 216-444-3279; E-mail: starkg@cesmtp.ccf.org.**

**The abbreviations used are: GH, growth hormone; GHR, GH receptor; STAT, signal transducer and activator of transcription; JAK, Janus kinase; IFN, interferon; IL, interleukin; EGF, epidermal growth factor; GAS, IFN-γ-activated sequence.**

---

* D. Watling, G. R. Stark, and I. M. Kerr, unpublished data.
GH Activation of JAKs and STATs

RESULTS

Expression of Functional GHR—The parental cell lines 2TGH and 2C4 and the mutant cell lines derived from them do not express endogenous GHR, as determined by FACSscan analysis (Fig. 1 and data not shown). Therefore, each cell line was transfected with a cDNA encoding wild-type human GHR under the control of a cytomegalovirus promoter (38). After selection for stable transfectants, the cells were sorted in order to obtain populations that expressed high levels of GHR (Fig. 1). GH stimulation of human GHR-transfected 2C4 cells (2C4/GHR) induced the phosphorylation on tyrosine of several cellular proteins with apparent molecular masses between 130–134 and 90–95 kDa (data not shown), indicating that the transfected cells did express functional receptor (39).

GH has been shown previously to promote phosphorylation of the receptor-associated tyrosine kinase JAK2 in a ligand-dependent manner (12). As shown in Fig. 2A, JAK2 (120 kDa) did become phosphorylated on tyrosine in 2C4/GHR cells following treatment with GH or IFN-α, but not IFN-α. JAK1 was also phosphorylated on tyrosine following GH stimulation, but as strongly as in cells treated with IFN-α or IFN-γ (Fig. 2B). These data indicate that JAK1 and JAK2 might both be involved in GH signaling in 2C4/GHR cells. Similar results were obtained with 3T3 F442A cells (Fig. 2, C and D). Treatment of cells with either IFN-α or IFN-γ leads to interdependent activation of two JAK family members (26, 27). To test whether the GH-dependent JAK1 and JAK2 phosphorylations were also interdependent, the phosphorylation of each JAK kinase was examined in mutant cells defective in the other. As shown in Fig. 2E, JAK2 activation in response to GH was normal in U4C/GHR cells (missing JAK1), in contrast to the result with IFN-γ, where JAK2 phosphorylation required JAK1 (27). The reciprocal experiment was performed in γ2A/GHR cells (missing JAK2). In this case, JAK1 was not phosphorylated on tyrosine in response to either GH or IFN-γ (Fig. 2F). Therefore, the phosphorylation of JAK1 by GH depends upon JAK2, but not vice versa.

GH Activation of STAT Proteins—STAT1 has been shown by some (9, 17), but not others (10, 11), to be activated in response to GH. In 2C4/GHR cells, STAT1 was strongly phosphorylated on tyrosine in response to GH, comparable to its phosphorylation in response to IFN-α or IFN-γ (Fig. 3A). We also examined other STAT proteins, including STAT3 (Fig. 3), STAT2, and STAT6. Of these, only STAT3 was phosphorylated on tyrosine in response to GH in both parental (Fig. 3B) and U3A/GHR (missing STAT1) (Fig. 3C) cells.

Mutant U4C/GHR and γ2A/GHR cells (missing JAK1 and JAK2, respectively) were used to examine the requirement for each JAK protein in GH-induced STAT activation. Treatments with IFN-α and IFN-γ were performed in parallel as controls. JAK2 was absolutely required for GH- and IFN-γ-dependent STAT activation (Fig. 3, F and G). JAK1, on the other hand, although phosphorylated in response to GH, was not required for GH-dependent STAT1 or STAT3 phosphorylation (Fig. 3, D and E). As expected, however, it was required for STAT activation by both IFN-α and IFN-γ (Fig. 3, D and E).

To extend these studies, STAT activation by GH was also examined by electrophoretic mobility shift assay in 2TGH/GHR and mutant cells. As shown in Fig. 4, three GH-induced complexes (A, B, and C) were detected with a 32P-labeled Fcy GAS probe in 2TGH/GHR, 2C4/GHR, and U4C/GHR cells, but not in γ2A/GHR cells. When γ2A/GHR cells were transfected with a murine JAK2 expression plasmid, STAT binding to the Fcy GAS probe was restored (albeit not to wild-type levels, probably due to poor JAK2 expression) (data not shown). The intensity of induced bands obtained in U4C/GHR cells was generally less than in other cell lines, but remained unchanged.

Electrophoretic Mobility Shift Assays—Cytoplasmic extracts, prepared as described by Sadowski and Gilman (35), were assayed with 32P-labeled oligonucleotides corresponding to the GAS elements of the Fcγ receptor factor I gene (Fcγ GAS) (36), the bovine casein gene (casein GAS) (31), or the high affinity c-sis-inducible element of the c-fos gene (37). For supershift analyses, the extracts were incubated with preimmune sera or specific STAT antisera for 30 min on ice before adding the labeled probes.
in U4C/GHR cells complemented with JAK1 (data not shown).

In U3A/GHR cells, only complex A was observed, suggesting that complexes B and C contain STAT1. Complexes A, B, and C were observed at normal levels in cells lacking STAT2 or Tyk2 (data not shown).

To analyze the complexes in more detail, antibody supershift experiments were performed (Fig. 5). As expected from the results with U3A/GHR cells, complexes B and C were lost when anti-STAT1 was included in the binding reaction (Fig. 5A, lane 3). Bands A and B were supershifted by anti-STAT3 (lane 5), suggesting that band B is a STAT1-STAT3 heterodimer and showing that band A contains STAT3; it is probably a homodimer. Antisera to STAT2, STAT5, or STAT6 had no effect when used with extracts from GH-treated 2fTGH/GHR cells (lanes 4, 6, and 7). In murine 3T3 F442A cells, four complexes were obtained with an Fcγ GAS probe. The lower three behaved identically to the three complexes observed in 2fTGH/GHR cells and represented STAT3 homodimer, STAT1-STAT3 heterodimer, and STAT1 homodimer, respectively. The uppermost band, however, was supershifted by anti-STAT5 (lane 14). To investigate further the possibility that STAT5 might be induced by GH in these cells, a GAS-like DNA-binding element from the promoter of the prolactin-responsive β-casein gene was used in band shift experiments (Fig. 5B). This probe has been shown to recognize STAT5 homodimers preferentially, but can also bind weakly to other STATs (31). The predominant complex obtained with extracts of 3T3 F442A cells was supershifted by anti-STAT5 (lane 3), but none of the complexes from 2TGH/GHR cells were affected by this antibody (lane 6). These data demonstrate that, in 3T3 F442A but not 2TGH/GHR cells, STAT5 or related proteins are activated and may be involved in GH signaling.

GH-induced Tyrosine Phosphorylation of GHR and Shc Requires JAK2—The human GHR, a transmembrane glycoprotein of ~134 kDa, becomes phosphorylated in response to GH, but has no intrinsic catalytic activity (40, 41). Although JAK2 is associated with the GHR, it is not clear whether it is required for receptor phosphorylation (12). To examine this possibility, cell lysates from GH-treated and untreated 2C4, 2C4/GHR, U4C/GHR, and γ2A/GHR cells were immunoprecipitated with anti-GHR monoclonal antibody 263 and then analyzed with
Fig. 5. Antibody supershift analysis of GH-induced STAT binding to a Fcγ GAS probe. A, whole cell extracts from untreated (lanes 1 and 9) or GH-treated (lanes 2–8 and 11–14) 2TGH/GHR cells (lanes 1–8) or 3T3 F442A cells (lanes 9–14) were analyzed. The STATs present in complexes were evaluated with antisera to STAT1 (lanes 3 and 12), STAT2 (lane 4), STAT3 (lanes 5 and 13), STAT5 (lanes 6 and 14), or STAT6 (lane 7) or with preimmune serum (PI; lane 8). A shorter exposure of the 3T3 F442A experiment is included to show the separation of the two upper complexes. The positions of the three GH-induced complexes observed in 2TGH/GHR cells are indicated, and the position of the GH-dependent complex obtained only in 3T3 F442A cells is marked with arrows. B, GH-dependent complexes formed with a casein GAS probe are shown. Binding assays were performed as described for A, using whole cell extracts from untreated (−) or GH-treated 3T3 F442A cells (lanes 1–3) or 2TGH/GHR cells (lanes 4–6). Supershift analysis was performed with a STAT5 antisera (lanes 3 and 6). AB, antibody; rhGH, recombinant human GH.

Fig. 6. Requirement of JAK2 for GH-dependent GHR phosphorylation. 2C4, 2C4/GHR, U4C/GHR, or γ2A/GHR cells were analyzed either without treatment (lanes 2, 4, and 6) or after treatment for 15 min with 500 ng/ml GH at 37 °C (lanes 1, 3, 5, and 7). Cell lysates were immunoprecipitated (IP) with a monoclonal antibody to the human GHR, and tyrosine phosphorylation was determined by Western analysis with anti-phosphotyrosine antibodies. The positions of molecular mass standards and of the IgG heavy chain (HC) are indicated. rhGH, recombinant human GH.

DISCUSSION

Using cell lines missing individual JAKs or STATs to examine their roles in GH signaling, we have shown that JAK2 is required for phosphorylation on tyrosine residues of STAT1 and STAT3, GHR, JAK1, and Shc. JAK2 has been implicated previously in signal transduction by GH, based on its association with the GHR and its activation upon ligand binding (12). However, it is clear that these criteria alone do not provide conclusive evidence that a given JAK has a functional role in a signal transduction pathway. For example, Tyk2, JAK1, and JAK2 are all phosphorylated in response to IL-6 (42, 43). However, experiments with mutant cells have demonstrated that only JAK1 is efficient in IL-6-dependent phosphorylation of the gp130 receptor subunit, STAT activation, and transcriptional induction of the interferon regulatory factor-1 gene (24). Furthermore, JAK1 is also phosphorylated in response to EGF, but is not required for STAT activation by this ligand (25). The data reported here suggest a similar situation for GH. JAK1 is phosphorylated on tyrosine in response to GH treatment of both 2C4/GHR and 3T3 F442A cells, a process that requires the presence of JAK2. However, STAT activation and ligand-dependent phosphorylation of the GHR and Shc were only slightly reduced in JAK1-minus U4C/GHR cells when compared with parental cells. It is unclear whether the reduced responsiveness to GH results from the JAK1 defect in these cells or whether other mutations contribute to this phenotype. Coimplementation with a murine JAK1 expression plasmid did not restore a wild-type response, and so it is assumed that JAK1 is dispensable for most, if not all, GH responses. Nevertheless, an involvement in some other aspect of GH signaling cannot be ruled out.

STAT5 has recently been shown to be activated in response to GH in GHR-transfected COS cells (19). That STAT5 or a
related factor is involved in GH signaling in some cell types is also demonstrated by our studies. STAT5, initially identified as a mediator of prolactin signaling in sheep (31), has since been implicated in erythropoietin (19) and IL-3 (44, 45) signal transduction. In the mouse, STAT5 is encoded by two genes that give rise to at least four different isoforms of STAT5 protein (44, 45). It is likely that the STAT5-like factor(s) that we have observed in 3T3 F442A cells represents one or more of these isoforms. Curiously, activation of STAT5 by GH was not observed in the human fibrosarcoma cells examined here, even though these cells express the STAT5 protein. It is unclear at this time whether the activation of STAT5 by GH is cell type-specific or whether species differences account for the difference between murine 3T3 F442A and human HT1080 cells. Cell type-restricted STAT activation has been described by others studying GH signaling. STAT1 is activated by GH in preadipocyte cells (Ref. 9 and this report) and rat liver (17), but not in IM9 lymphoblastoid cells (10, 11). Thus, it appears that selectivity involving specific STAT subsets is a general feature of GH signal transduction.

The mechanism of STAT activation by GH is not well understood. Elimination of all sites for tyrosine phosphorylation from the intracellular domain of the GHR did not prevent signaling or STAT activation (13), arguing against a direct, SH2-mediated interaction between STATs and the GHR. In contrast, STAT1 interacts with the specific phosphoryrosine Tyr(P)

\( ^{405} \) of the IFN-γ receptor, which lies in the sequence GpYDKPH (46), and STAT3 binds to the intracellular domain of the gp130 receptor, where the sequence motif pYXXQ (X is any amino acid) appears to be both necessary and sufficient for STAT-receptor interaction (43). Neither of these sequences is present in the GHR. Therefore, STAT interaction with the GHR may be mediated either through adaptor molecules or through a Jak2-STAT association. Alternatively, the GHR may require additional receptor subunits, as yet unidentified, that function in STAT binding.

Our data indicate that, in addition to its important role in coupling the GHR to STAT signaling pathways, Jak2 is required to couple the receptor to pathways involving Shc. Shc is thought to function as an adaptor molecule to recruit Grb2-msos1 complexes to the activated receptor (47). The nucleotide exchange factor msos1 then promotes formation of p21

\( ^{Ras(GTP)} \), initiating a cascade of phosphorylation events that culminate with phosphorylation of specific transcription factors in the nucleus (16, 47). In EGF signaling, Shc interacts with the EGF receptor by binding to specific phosphotyrosine residues within its cytoplasmic tail (48, 49). In the case of erythropoietin, it has been reported that receptor phosphorylation is not required for ligand-dependent Shc-receptor association or for Shc phosphorylation (50). Instead, Shc appears to associate directly with Jak2 following erythropoietin treatment (50). A similar situation may exist for GH since Jak2 and Shc become associated following GH stimulation (14). These data suggest that Shc may be a direct target for phosphorylation by Jak2 following GH and erythropoietin stimulation, although in the case of GH, it is currently unknown whether receptor phosphorylation is required for Shc-GHR interaction.

Further insight into GH signaling should be obtained when more genes that are targets for GH activation are identified. Although several early response genes, such as c-fos, are known to be transcriptionally activated by GH (51), the mechanism of induction is not known. The c-fos promoter contains the cis-inducible element, a canonical STAT binding sequence thought to play a role in induction by c-sis (platelet-derived growth factor) (37, 52). However, the c-fos promoter is complex, and its transcriptional regulation involves coordinated activation through elements that respond to both Ras-dependent and Ras-independent pathways (16, 53). Therefore, the identification of GH-responsive genes that respond exclusively through the Jak-STAT pathway, if they exist, would provide a better means to assess the roles of individual STATs in the transcriptional response to GH.

Acknowledgments—We thank Drs. A. Ziemiecki, J. Ihle, J. Darnell, Jr., and D. Levy for the gifts of specific JAK and STAT antisera.

REFERENCES

1. Isaksson, O. G. P., Eden, S., and Jansson, J.-O. (1985) Annu. Rev. Physiol. 47, 483–499
2. Kishimoto, T., Taga, T., and Akira, S. (1994) Cell 76, 253–262
3. Ihle, J. N., Wittlhuhn, B. A., Quelle, F. W., Yamamoto, K., Trethewer, W. E., Kreider, B., and Silvennoinen, O. (1994) Trends Biochem. Sci. 19, 222–227
4. Ihle, J., and Kerr, I. M. (1995) Trends Genet. 11, 69–74
5. Bazan, J. F. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 6934–6938
6. Stred, S. E., Stubbart, J. R., Argetsinger, L. S., Smith, W. C., Shafer, J. A., Talamantes, F., and Carter-Su, C. (1992) Endocrinology 130, 1626–1636
7. Darnell, J. E., Jr., Kerr, I. M., and Stark, G. R. (1994) Science 264, 1415–1421
8. Martin, S., Nisell, G., and Groner, B. (1994) EMBO J. 13, 27532–27539
9. Argetsinger, L. S., Campbell, G. S., Cochran, B. H., Argretinger, L. S., Lerner, A. C., Finlons, D. S., Carter-Su, C., and Schwartz, J. (1994) J. Biol. Chem. 269, 4701–4704
10. Finlons, D. S., Petricoin, E. F., III, Hackett, R. H., David, M., Feldman, G. M., Igarashi, K., Fibach, E., Weber, M. J., Thorton, M. E., Silva, C. M., and Lerner, A. C. (1994) Mol. Cell. Biol. 14, 2113–2118
11. Silva, C. M., Weber, M. J., and Thorton, M. O. (1994) J. Biol. Chem. 269, 27532–27539
12. Argetsinger, L. S., Campbell, G. S., Yang, X., Wittlhuhn, B. A., Silvennoinen, O., Ihle, J. N., and Carter-Su, C. (1993) Cell 74, 237–244
13. Wang Y.-D., Wong, K., and Wood, W. I. (1990) J. Biol. Chem. 270, 7021–7024
14. VanderKuik, J., Allegrato, G., Billestrup, N., Norstedt, G., and Carter-Su, C. (1995) J. Biol. Chem. 270, 7587–7593
15. Veisz, A. R., Schindler, C., Leonard D., Fu, X.-Y., Aebisold, R., Darnell, J. E., Jr., and Levy, D. E. (1992) Mol. Cell. Biol. 12, 3315–3324
16. Hill, C. S., and Treisman, R. (1995) Cell 80, 199–211
17. Gronowski, A. M., and Rotwein, P. (1994) J. Biol. Chem. 269, 7874–7878
18. Campbell, G. S., Meyer, D. J., Raz, R., Levy, D. E., Schwartz, J. Z., and Carter-Su, C. (1995) J. Biol. Chem. 270, 3974–3979
19. Gouilleux, F., Pallard, C., Dusander-Fourt, I., Wakao, H., Haldosen, L.-A., Norstedt, G., Levy, D. E., and Groner, B. (1995) EMBO J. 14, 2005–2013
20. Thomas, M. J., Gronowski, A. M., Berry, S. A., Bergland, P. L., and Rotwein, P. (1995) Mol. Cell. Biol. 15, 12–18
21. Pellegrini, S. John J., Shearer, M. J., and Stark, G. R. (1993) Mol. Cell. Biol. 13, 4189–4198
22. McKendry, R., John J., and Flavel, M. R., Kerr, I. M., and Stark, G. R. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 11455–11459
23. Guschin, D., Rogers, N., Briscoe, J., Witthuhn, B., Watling, D., Horn, F., Pellegrini, S., Yasukawa, K., Heinrich, P., Stark, G. R., Ihle, J. N., and Kerr, I. M. (1995) EMBO J. 14, 1421–1429
24. Leman, D., Pichardy, S., Flickett, T. W., Commene, M. A., Schleisinger, J., Kerr, I. M., Levy, D. E., and Stark, G. R. (1995) Mol. Cell. Biol. 16, 3619–375
