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Regulation of Glucose Transport and c-*fos* and *egr-1* Expression in Cells with Mutated or Endogenous Growth Hormone Receptors*

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ABSTRACT

To identify mechanisms by which GH receptors (GHR) mediate downstream events representative of growth and metabolic responses to GH, stimulation by GH of c-*fos* and *egr-1* expression and glucose transport activity were examined in Chinese hamster ovary (CHO) cells expressing mutated GHR. In CHO cells expressing wild-type GHR (GHR_{1-638}), GH stimulated the expression of c-*fos* and *egr-1*, and stimulated 2-deoxyglucose uptake, responses also mediated by endogenous GHR in 3T3-F442A cells. Deletion of the proline-rich box 1 of GHR ($GHR_{\Delta P}$) abrogated all of these responses to GH, indicating that box 1, a site of association of GHR with the tyrosine kinase JAK2, is crucial for these GH-stimulated responses. As the C-terminal half of the cytoplasmic domain of GHR is required for GH-stimulated calcium flux and for stimulation of *spi-2.1* transcription, GHR lacking this sequence (GHR_{1-454}) were examined. Not only did GHR_{1-454} mediate stimulation of c-*fos* and *egr-1* expression and 2-deoxyglucose uptake, but they also mediated GH-stimulated transcriptional activation via Elk-1, a transcription factor associated with the c-*fos* Serum Response Element. Thus, the C-terminal half of the cytoplasmic domain of GHR is not required for GH-stimulated c-*fos* transcription, suggesting that increased calcium is not required for GH-stimulated c-*fos* expression. In CHO cells lacking all but five N-terminal residues of the cytoplasmic domain (GHR_{1-294}), GH did not induce c-*fos* or *egr-1* expression or stimulate 2-deoxyglucose uptake. Further, in 3T3-F442A fibroblasts with endogenous GHR, GH-stimulated c-*fos* expression and 2-deoxyglucose uptake were reduced by the tyrosine kinase inhibitors herbimycin A, staurosporine, and P11. Herbimycin A and staurosporine inhibit JAK2 and tyrosyl phosphorylation of all proteins stimulated by GH, whereas P11 inhibits the GH-dependent tyrosyl phosphorylation of only some proteins, including extracellular signal regulated kinases ERK1 and -2, but not JAK2. Taken together, these results implicate association of GHR with JAK2 and GH-stimulated tyrosyl phosphorylation of an additional cellular protein in GH-stimulated glucose transport and c-*fos* and *egr-1* expression. These studies also indicate that, in contrast to *spi-2.1*, the N-terminal half of the cytoplasmic domain of GHR is sufficient to mediate stimulation of c-*fos* and *egr-1* expression and Elk-1 activation, supporting multiple mechanisms for GH signaling to the nucleus. (*Endocrinology* **139:** 1863–1871, 1998)

THE DIVERSE effects of GH on cell growth, differentiation, and metabolism are thought to share early signaling pathways involving the GH receptor (GHR). The GHR is a single transmembrane protein with an extracellular binding domain and a cytoplasmic domain that mediates signaling (1). For insight into GH signaling mechanisms, functions of the GHR have been dissected by analysis of GHR mutants in which the cytoplasmic domain has been truncated or mutated (2–10). A complex picture is emerging indicating that the N-terminal half of the cytoplasmic domain of GHR is sufficient to initiate some GH-stimulated events, such as stimulation of protein and lipid synthesis, but that the C-terminal half of the cytoplasmic domain is additionally required for other responses to GH, including activation of the *spi-2.1* gene and stimulation of insulin synthesis (1). Activation of JAK2, via association with box 1 just proximal to the transmembrane domain of GHR (10, 11), is required for most, but not all, responses to GH identified to date, including tyrosyl phosphorylation of signaling molecules such as SHC, a component of the Ras-mitogen-activated protein kinase (MAPK) pathway (12–16), insulin receptor substrate-1 (IRS-1) and- 2 (17–19), and signal transducers and activators of transcription-1 (Stats), 1, -3, -5A, and -5B (8, 20–22), as well as induction of *spi-2.1* gene expression. Interestingly, stimulation of calcium flux by GH appears to be independent of JAK2, as it is reported to be mediated by GHR lacking a functional box 1 (4).

The present study examines the ability of mutated and truncated GHR to mediate GH responses thought to be rep-

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resentative of its growth and metabolic effects. GH-regulated early response genes such as c-*fos* have been implicated in growth regulatory events. Stimulation of c-*fos* occurs in response to a wide variety of agents and can be mediated by various upstream regulatory sequences, including those regulated via ERKs, Stat1 and -3, or calcium (23, 24), making it useful for assessing GH signaling and potentially identifying JAK2-dependent or -independent mechanisms. Analysis of GHR requirements for induction of c-*fos* is also revealing by comparison with the *spi-2.1* gene, which is stimulated by GH through γ-activated sequence-like elements (GLE) that bind Stat5; such stimulation requires both N- and C-terminal regions of the cytoplasmic domain of GHR (4). In c-*fos*, the Serum Response Element (SRE) can mediate induction by GH (25), and SRE-associated transcription factors Elk-1 and serum response factor are required for such induction (26). The evaluation presented herein of the ability of GHR to mediate expression of SRE-containing genes such as c-*fos* and *egr-1* (27–30), and transcriptional activation via Elk-1 can thus provide insight into whether GHR requirements are similar for regulating different genes that use different transcription factors. As a representative metabolic response to GH, the regulation of glucose transport, a rate-limiting step in cellular carbohydrate metabolism and an important determinant in the ability of GH to regulate carbohydrate and lipid metabolism (31, 32), was examined. Responses to GH were assessed in Chinese hamster ovary (CHO) cells expressing wild-type or mutated GHR. Signaling mechanisms elicited by the endogenous GHR were also probed using 3T3-F442A fibroblasts treated with a panel of tyrosine kinase inhibitors.

Materials and Methods

Materials

CHO cells expressing wild-type or mutated rat GHR have been described previously (10, 15). A stock of 3T3-F442A fibroblasts was provided by Dr. H. Green, Harvard University (Boston, MA). Human GH prepared by recombinant DNA techniques was provided by Genentech (South San Francisco, CA) and Eli Lilly Co. (Indianapolis, IN). Herbimycin A was a gift from the NCI. Staurosporine was purchased from Boehringer Mannheim (Indianapolis, IN). The inhibitor P11, (acetyloxy)methoxy-(2-naphthyl) methylphosphonic acid bis(acetoxy methyl)ester, a membrane-permeant derivative of hydroxy-(2-naphthyl)methylphosphonic acid, was a gift from Merck, Sharpe, and Dohme (Rahway, NJ). DMEM, Ham's F-12 medium, L-glutamine, antibiotic-antimycotic solution, calf serum, and FBS were purchased from Irvine Scientific (Santa Ana, CA). BSA (CRG-7) was purchased from Intergen Pharmaceuticals (Purchase, NY). 2-Deoxy-D-glucose, phloretin, β-mercaptoethanol, and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{32}P]Deoxy-CTP and 2-deoxy-D-[1-^{14}C]glucose were purchased from Sigma or DuPont-New England Nuclear (Boston, MA). Random priming kits were purchased from Life Technologies (Gaithersberg, MD).

Culture and treatment of cells expressing GHR

GHR variants were assessed in CHO cells stably transfected with mammalian expression vectors containing the complementary DNA (cDNA) encoding the full-length rat liver GHR (GHR_{1-638}) sequence, truncated GHR sequences with stop codons at positions 455 (GHR_{1-454}) or 295 (GHR_{1-294}), as described previously (15, 33). The same full-length GHR cDNA was mutated to delete box 1 by deleting the codons for amino acids at positions 297–311 ($GHR_{\Delta P}$). CHO cells expressing full-length, truncated, or mutated GHR were maintained as described previously (10, 34). The relative levels of [^{125}I]human GH binding among the cell lines were comparable to those reported previously (8, 10, 15). Confluent CHO cells were deprived of serum overnight by incubating cells in Ham's F-12 medium containing 1% BSA. Cells were then treated with or without 500 ng/ml (22 nM) GH for 30 min, unless indicated otherwise, and used for analysis of GH activity.

To assess the function of endogenous GHR, 3T3-F442A preadipocyte fibroblasts were grown to confluence as described previously (35). In experiments using tyrosine kinase inhibitors, 3T3-F442A fibroblasts were deprived of serum overnight in DMEM containing 1% BSA. Cells were preincubated during the deprivation period with one of the inhibitors, herbimycin A (1.8 μM; for 18–22 h), P11 (100 μM; for the final 1 h), staurosporine (500 nM; for the final 1 h or 10 min), or vehicle (DMSO). The conditions used were those established previously to interfere with GH-dependent tyrosyl phosphorylation of cellular proteins (12), except that when glucose uptake was measured, cells were preincubated with staurosporine for 1 h instead of 10 min. Immunoblotting of whole cell lysates with antiphosphotyrosine antibody confirmed that the inhibitors reduced tyrosyl phosphorylation of JAK2 and other cellular proteins as described previously (12). GH (500 ng/ml; 22 nM) was added in the presence of inhibitors for 30 min, unless indicated otherwise, and cells were used for analysis of c-*fos* expression or measurement of 2-deoxyglucose uptake.

Preparation of total RNA and Northern blot analysis

After treatment, the 3T3-F442A or CHO cells were washed with PBS, and RNA was prepared by the acid phenol/guanidine isothiocyanate method (36). Total RNA was fractionated on a 1% agarose-formaldehyde gel and transferred to nitrocellulose or Nytran (Schleicher and Schuell, Keene, NH). The membranes were hybridized with mouse c-*fos* (35) or mouse *egr-1* (37) probes labeled with [^{32}P]deoxy-CTP by random priming (35). The *egr-1* cDNA was provided by Dr. L. Lau (University of Illinois, Chicago, IL).

Elk-1-mediated transcriptional activation

For analysis of transcriptional activation via Elk-1, the expression plasmid Gal4/ElkC (38), encoding the transcriptional activation domain of Elk-1 fused to the Gal4 DNA-binding domain, and the reporter plasmid 5X Gal/Luc (39), containing five consensus Gal4 DNA-binding sites upstream of the luciferase gene, were provided by Dr. C. Der (University of North Carolina, Chapel Hill, NC). CHO cells expressing the indicated GHR (2×10^5 cells/35 mm well) were cotransfected with 5 μg/well Gal4/ElkC and Gal/Luc DNA, using calcium phosphate (40). Forty-four to 48 h after transfection, cells were deprived of serum and treated with or without GH for 4 h. Cells were then lysed in reporter lysis buffer (100 mM potassium phosphate, 0.2% Triton X-100, and 1 mM dithiothreitol), and luciferase activity was measured using an Autolumat Luminometer (Wallac-Berthold, Gaithersberg, MD).

Glucose transport assay

Glucose transport was assayed by measurement of 2-deoxy-D-glucose uptake. Where specified, cells were incubated with the indicated inhibitor or corresponding vehicle in 1% BSA-Ham's F-12 medium (CHO cells) or with 1% BSA in DMEM (3T3-F442A fibroblasts), followed by 30-min incubation with vehicle (control) or with GH (500 ng/ml) in Krebs-Ringer phosphate buffer (KRP) containing 1% BSA. The glucose transport assay was initiated by incubating cells at 37 C with fresh KRP-BSA plus 100 μM unlabeled 2-deoxyglucose and 3.4 μM [^{14}C]2-deoxyglucose. In 3T3-F442A fibroblasts, the assay was terminated after 5 min by aspiration of the 2-deoxyglucose solution. In CHO cells, the time of incubation with radioactive tracer was extended to 20 min because the amount of Glut1 glucose transporter present in these cells is very low (41). Cells were washed twice with ice-cold KRP containing phloretin (200 μM) to block further uptake of 2-deoxyglucose and were scraped in 0.1% SDS. Aliquots of the cell lysates were used to assess radioactivity and protein content (42). Glucose transport was calculated as picomoles of 2-deoxyglucose transported per mg protein/min. Each condition was tested in triplicate in each experiment, and values for the increments due to GH are presented as mean ± SE for replicate experiments. Data were analyzed by Student's *t* test. As established previously for 3T3-F442A cells (32, 43), GH-stimulated 2-deoxyglucose uptake was inhibited by cytochalasin B and was linear for the duration of

the assay in the CHO cells (not shown). In addition, 2-deoxyglucose uptake was stimulated independently by insulin (1 μg/ml) and phorbol dibutyrate (500 ng/ml), an activator of protein kinase C (PKC),[1] providing evidence for a functional glucose transport mechanism in CHO cells.

Results

Stimulation of c-fos and egr-1 expression is abrogated by deletion of box 1 of GHR

CHO cells expressing GHR mutants have been characterized for their ability to mediate a variety of responses, including association with and activation of JAK2, activation of MAPK, transcription of the *spi-2.1* gene and calcium oscillations (4, 10, 15, 44, 45). The GHR mutants used in this study and their ability to mediate some GH-dependent signaling events are summarized in Fig. 1.

In CHO cells expressing full-length GHR (GHR_{1-638}), GH clearly induced the expression of c-*fos* (Fig 2A, lane 4, *top*; and Fig. 2B, lane 2), whereas the parent CHO cells not expressing GHR failed to respond to GH (Fig. 2A, lane 2). The expression of *egr-1*, another early response gene, was also stimulated via the full length GHR (Fig. 2A, lane 4, *middle*; Fig 2C, lane 2). The fact that CHO cells expressing GHR exhibit these responses to GH indicates that the intracellular components essential in GH signaling to stimulate early response gene expression are present in CHO cells. A GH-induced increase in *egr-1* was also mediated by endogenous GHR in 3T3-F442A cells (Fig. 3), which showed a time course and dose dependence similar to that for c-*fos* (35).

A proline-rich motif (box 1), which in GHR is just C-terminal of the transmembrane domain, is found in all members of the cytokine/hematopoietin receptor superfamily (46) and has been found to be necessary for association with and activation of JAK2 (10, 11). Deletion of the proline-rich region from the full-length GHR ($GHR_{\Delta P}$) interfered with its ability to mediate c-*fos* or *egr-1* induction in response to GH compared with the full-length receptor (Fig. 2A, lane 6). The lack of response with $GHR_{\Delta P}$ supports the necessity for the association of JAK2 with GHR for stimulation of c-*fos* and *egr*-1 expression by GH. GHR in which all but five residues of the cytoplasmic domain were deleted (GHR_{1-294}) failed to mediate induction of c-*fos* or *egr-1* expression in response to GH (Fig. 2, B and C, lane 6), as it failed to elicit other early signaling events, including activation of JAK2 (16).

The C-terminal half of the cytoplasmic domain of GHR is not required for GH-stimulated early response gene expression

To examine whether the C-terminal region of GHR is required to mediate stimulation of c-*fos* and *egr-1* expression, as it is for *spi-2.1* expression and for increased intracellular calcium, cells expressing GHR in which the C-terminal half of the cytoplasmic domain was deleted (GHR_{1-454}) were studied. Truncation of the C-terminal half of the cytoplasmic domain of GHR did not interfere with induction of c-*fos* or *egr-1* expression by GH (Fig. 2, B and C, lane 4). Stimulation of c-*fos* and *egr-1* was consistently observed with both

[1] Gong, T. W., and J. Schwartz, unpublished observations.

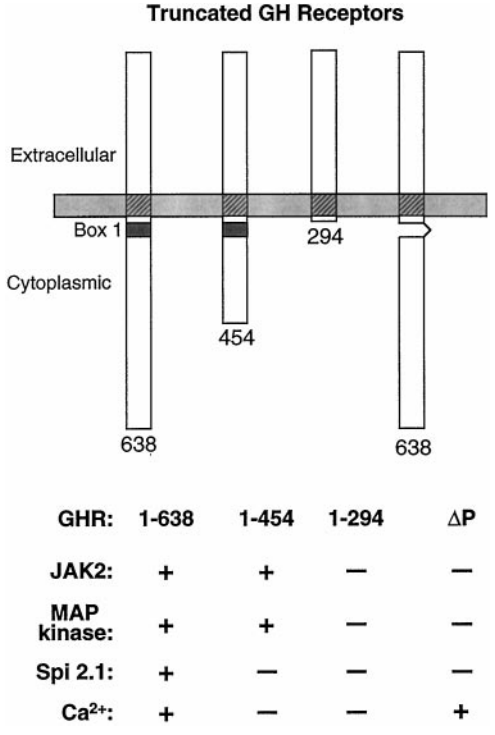

FIG. 1. Wild-type and mutated GHR expressed in CHO cells. The extracellular domain, the transmembrane domain (*hatched area*), and the cytoplasmic domain of expressed rat liver GHR are shown. Box 1 is indicated by the *shaded box*. +, Responses to GH mediated by GHR mutants; −, lack of response. Adapted in part from Ref. 10.

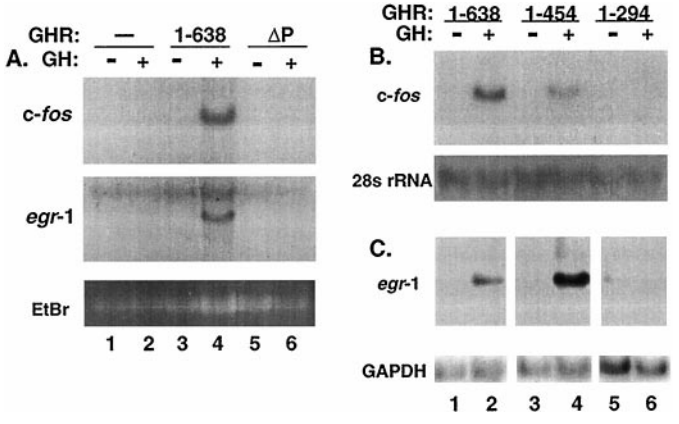

FIG. 2. Induction of c-*fos* and *egr-1* expression by GH in CHO cells expressing mutated GHR. Effect of GH on c-*fos* or *egr-1* expression in A) untransfected CHO cells (−) and CHO cells stably expressing GHR_{1-638} or $GHR_{\Delta P}$, and B and C) cells expressing GHR_{1-638}, GHR_{1-454}, or GHR_{1-294}. Cells were incubated with or without GH for 30 min. Total RNA was prepared and subjected to Northern blot analysis. *Bottom panels* show expression of 28S ribosomal RNA or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by hybridization or ethidium bromide staining of the corresponding gel, indicating RNA loading. Each experiment was repeated 2–3 times.

GHR_{1-638} and GHR_{1-454}, although the relative intensity of the stimulation via each GHR was variable. Thus, the N-terminal half of the cytoplasmic domain of GHR appears to be sufficient for GH signaling to regulate c-*fos* and *egr-1* expression, distinguishing induction of c-*fos* and *egr-1* from that of *spi-2.1*,

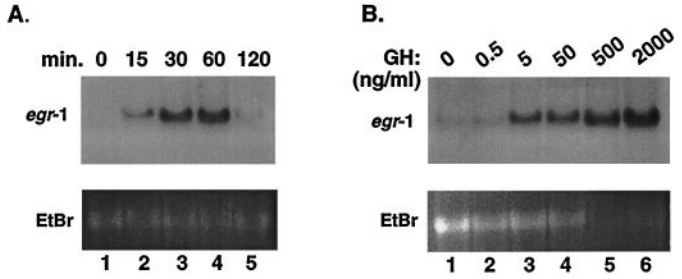

FIG. 3. GH rapidly and transiently stimulates the expression of *egr-1* in 3T3-F442A cells. A, Quiescent 3T3-F442A fibroblasts were incubated with GH (500 ng/ml) for 0–120 min. B, Quiescent 3T3-F442A fibroblasts were incubated for 30 min with the indicated concentrations of GH. Total RNA was prepared and subjected to Northern blot analysis using a mouse *egr-1* probe that detects a 3.4-kilobase transcript. The *bottom panels* show ethidium bromide staining of the corresponding gel. Each experiment was repeated one to three times.

and suggesting that increased intracellular calcium is not required for their expression.

GH-stimulated transcriptional activation mediated by Elk-1 does not require the C-terminal half of the cytoplasmic domain of GHR

In c-*fos*, the SRE can mediate induction by GH (25), and SRE-associated transcription factors Elk-1 and Serum Response Factor are required for such induction (26). For insight into how GHR might mediate transcriptional activation via the c-*fos* SRE, the ability of GHR lacking the C-terminal half of the cytoplasmic domain to mediate activation of transcription via Elk-1 was examined. Elk-1-mediated transcriptional activation was doubled by GH in CHO cells expressing either full-length GHR or GHR_{1-454} (Fig. 4), indicating that the C-terminal half of the cytoplasmic domain of GHR is not required for transcriptional activation via Elk-1 in response to GH. Basal transcription via Elk-1 was slightly lower with GHR_{1-454} for reasons that are not clear. GHR_{1-294} failed to mediate transcriptional activation via Elk-1 (Fig. 4) in response to GH. Thus, the N-terminal half of the cytoplasmic domain of GHR is sufficient to mediate GH-promoted c-*fos* expression and activation of Elk-1, consistent with a critical role for Elk-1 in GH-stimulated c-*fos* expression.

GH-stimulated glucose uptake requires box 1 but not the C-terminal half of the cytoplasmic domain of GHR

To determine what regions of GHR are required for GH-stimulated glucose uptake, 2-deoxyglucose uptake was measured in CHO cells expressing GHR variants (Fig. 5). GH stimulated 2-deoxyglucose uptake via the full-length GHR (GHR_{1-638}) in CHO cells. The relatively modest stimulation of glucose uptake by GH most likely reflects the low abundance of Glut1 and the absence of Glut4 glucose transporters in CHO cells (40, 41). The increment in glucose uptake in response to GH was statistically significant and reproducible and was clearly in contrast to the lack of response to GH treatment in untransfected parental CHO cells. Importantly, the stimulation of glucose transport by GH was not evident in cells expressing $GHR_{\Delta P}$, which lacks box 1, indicating the importance of the association of GHR and JAK2 for GH-stimulated glucose uptake. CHO cells expressing GHR_{1-454}

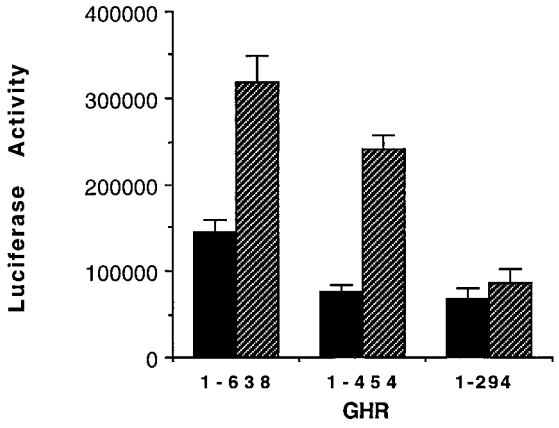

FIG. 4. Elk-1-mediated transcriptional activation in CHO cells expressing mutated GHR. CHO cells stably expressing GHR_{1-638}, GHR_{1-454}, or GHR_{1-294} were transiently transfected with ElkC/Gal and Gal/Luc. Two days later, they were treated without (*solid bar*) or with 500 ng/ml GH (*hatched bar*) for 4 h, and luciferase activity was measured. The experiment was performed twice. Representative data (mean ± SEM of triplicate observations) from one experiment are shown.

showed an increase in 2-deoxyglucose uptake in response to GH that was comparable to that with GHR_{1-638}, indicating that the N-terminal half of the cytoplasmic domain of the GHR is sufficient to mediate the stimulation of glucose transport by GH. GHR_{1-294} failed to mediate the stimulation of 2-deoxyglucose uptake by GH. These results indicate that the ability of GH to stimulate glucose transport, like induction of c-*fos* or *egr-1* expression, corresponds to the retention in GHR of the ability to associate with JAK2. They also indicate that the N-terminal half of the cytoplasmic domain of GHR is sufficient to mediate GH-stimulated glucose transport.

GH-induced c-fos expression and glucose transport are attenuated by tyrosine kinase inhibitors in 3T3-F442A cells

Endogenous GHR mediate tyrosyl phosphorylation of multiple proteins in 3T3-F442A cells (12). To relate tyrosyl phosphorylation and downstream responsiveness to GH via endogenous GHR, a panel of tyrosine kinase inhibitors was used under conditions previously established to interfere with GH-stimulated tyrosyl phosphorylation of JAK2 and/or other cellular proteins (12). Immunoblotting of lysates of 3T3-F442A cells with antiphosphotyrosine antibody confirmed in the present experiments that GH-dependent tyrosyl phosphorylation of cellular proteins, including JAK2 and ERK1 and -2, was diminished by herbimycin A and staurosporine (data not shown), as reported previously (12). The inhibitor P11 reduced tyrosyl phosphorylation of a subset of these proteins, including ERK1 and -2, but not JAK2, as previously reported (12). Under identical treatment conditions, the increase in c-*fos* messenger RNA induced by GH in control cells (Fig. 6, lane 2) was absent when herbimycin A, P11, or staurosporine was present (Fig. 6, lanes 4, 6, and 8, respectively), indicating that all three tyrosine kinase inhibitors interfered with GH-stimulated c-*fos* expression.

GH treatment alone consistently increased 2-deoxyglucose uptake to 3.5 times the control value in 3T3-F442A fibroblasts in these experiments (Fig. 7). Preincubation with herbimycin

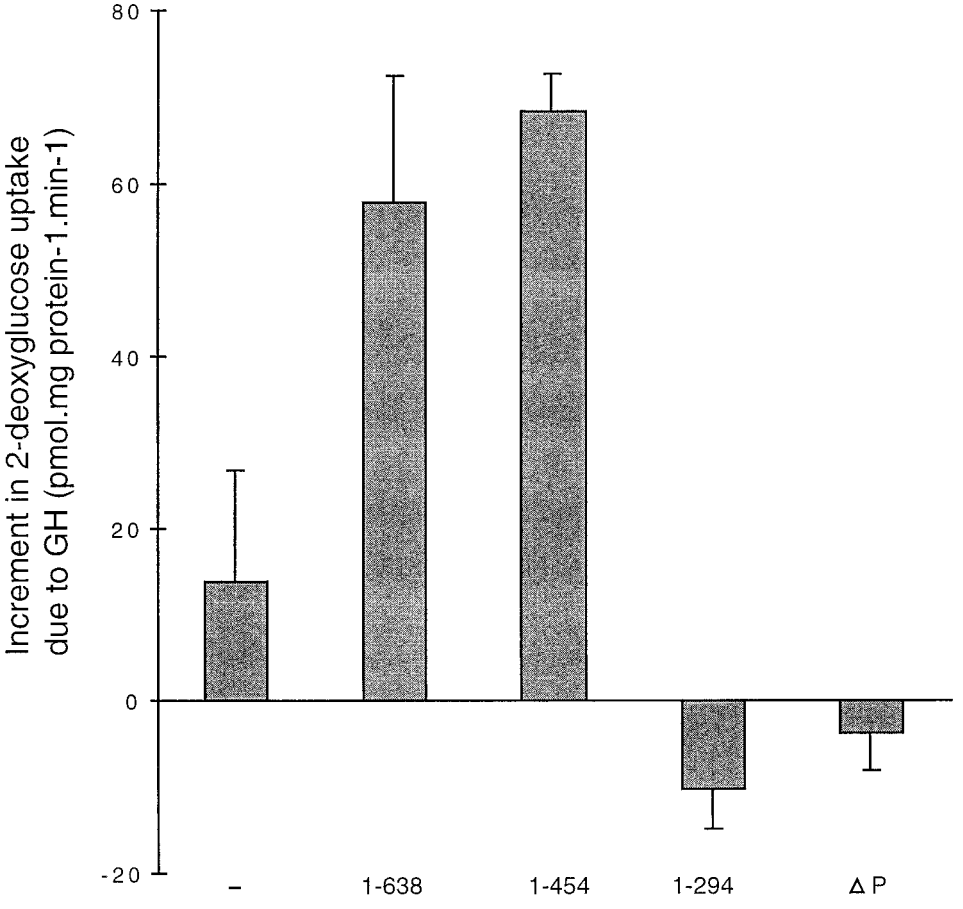

FIG. 5. Stimulation of glucose uptake by GH in CHO cells expressing mutated GHR. The increment in 2-deoxyglucose uptake due to GH, expressed in picomoles per mg protein/min, is shown for untransfected CHO cells (−) and CHO cells stably expressing GHR_{1-638}, GHR_{1-454}, GHR_{1-294}, or $GHR_{\Delta P}$. Cells were incubated with or without GH for 30 min before measurement of glucose uptake. *Bars* represent the mean ± SE for 3–4 independent experiments. Control glucose uptake values were 210 ± 110 for parental CHO cells, 210 ± 50 for GHR_{1-638}, 110 ± 30 for GHR_{1-454}, 340 ± 100 for GHR_{1-294}, and 175 ± 51 for $GHR_{\Delta P}$. The response to GH was significant for GHR_{1-638} ($P < 0.02$) and GHR_{1-454} ($P < 0.01$).

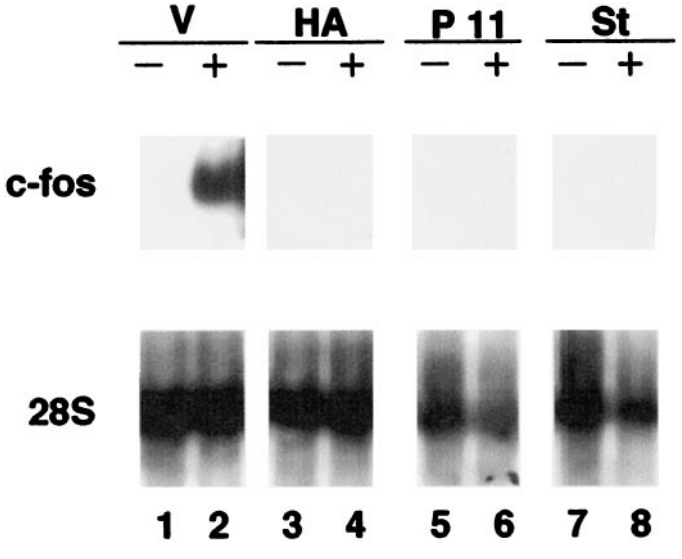

FIG. 6. Inhibition of GH-induced c-*fos* expression in 3T3-F442A cells in the presence of tyrosine kinase inhibitors. 3T3-F442A fibroblasts were treated with herbimycin A (HERB A), P11, staurosporine (St), or DMSO vehicle as described, followed by 30-min treatment without (C) or with GH. Northern blot analysis was performed using c-*fos* as probe (*top panel*). The *bottom panel* shows expression of 28S ribosomal RNA. This experiment was repeated twice.

A, staurosporine, or P11 diminished the stimulation by GH to similar extents (79%, 84%, and 62%, respectively; Fig. 7). In the adipocyte form of 3T3-F442A cells, degrees of inhibition comparable to those observed in the fibroblast form were obtained when GH-stimulated glucose transport was measured in the presence of the inhibitors (data not shown).

Discussion

The site of association of GHR with JAK2 is required for GH-stimulated c-fos and egr-1 expression and glucose uptake

The current model of GH signaling is that upon ligand binding, association of GHR and JAK2 initiates tyrosyl phosphorylation of JAK2 and GHR (47). Activated JAK2 then initiates a rapid sequence of events leading to the GH-promoted tyrosyl phosphorylation and activation of a variety of cellular proteins, including SHC, MAPK (12–16), IRS-1 and -2 (17–19), and Stats 1, -3, -5A, and -5B (8, 20–22). Each of these signaling molecules may participate in one or more subsequent biological events, thus contributing to multiple JAK2-dependent signaling pathways initiated via the GHR (1). Accordingly, failure of JAK2 to associate with GHR would be expected to interfere with subsequent JAK2-dependent events in GH action. Consistent with this, $GHR_{\Delta P}$ lacking box 1, the site of association with JAK2, fails to

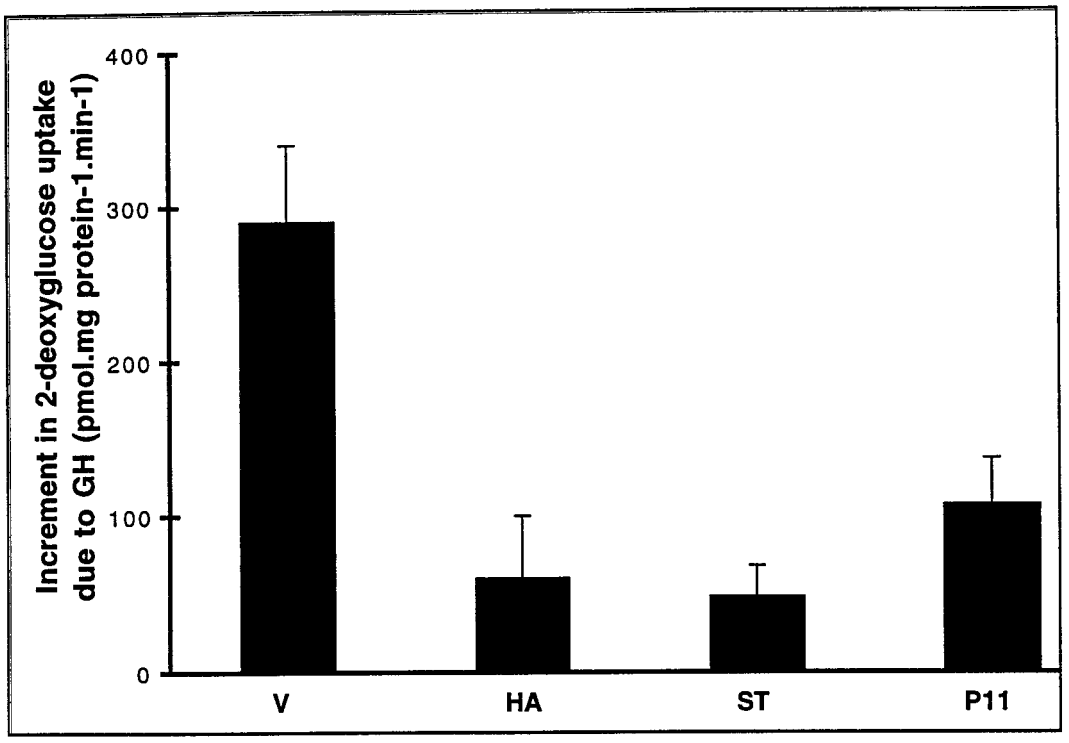

FIG. 7. Inhibition of GH-stimulated glucose uptake in 3T3-F442A cells in the presence of tyrosine kinase inhibitors. 3T3-F442A fibroblasts were preincubated with the indicated inhibitors, followed by a 30-min treatment with vehicle or GH, and 2-deoxyglucose uptake was measured. Increments in 2-deoxyglucose uptake due to GH are shown. *Bars* and control values are expressed as the mean + SE for three or four independent experiments, each performed in triplicate. Control values for glucose uptake were: DMSO, 110 ± 130; herbimycin A (HA), 150 ± 50; staurosporine (ST), 310 ± 20; and P11, 150 ± 40. The increment due to GH was significant in the presence of DMSO vehicle for the combined experiments ($P < 0.001$) and in the presence of P11 ($P < 0.05$).

mediate SHC, MAPK, IRS, or Stat activation (15–17, 48, 49). However, JAK2 is not implicated in all GHR-mediated events. GH-induced calcium oscillations are reported to occur in cells expressing $GHR_{\Delta P}$ and $GHR_{P>A}$ lacking a functional box 1 (4), suggesting a JAK2-independent GH signaling pathway for this response.

The results of this study are consistent with JAK2-dependent events mediating induction of c-*fos* and *egr-1* and stimulation of glucose uptake in response to GH. This role is supported by the observation that $GHR_{\Delta P}$ failed to mediate these responses, indicating that the site of association of JAK2 and GHR is required for the ability of GH to elicit these responses. Furthermore, the tyrosine kinase inhibitors staurosporine and herbimycin, which are known to inhibit JAK2, blocked all three responses. It is recognized that although the simplest interpretation of these findings is that JAK2 is involved, the alternative explanation, that conformational changes in GHR secondary to deletion of box 1 are involved in these responses cannot be ruled out. Furthermore, involvement of tyrosine kinases in addition to JAK2 is suggested below.

Signaling pathways mediating GH stimulated c-fos expression

Stimulated c-*fos* can be mediated by various upstream regulatory sequences, including those regulated via ERKs, Stat1 and -3, or calcium (23, 24). Several signaling pathways converge on the SRE (23), which is known to mediate stimulation of c-*fos* by GH (25, 26, 50, 51). Several lines of evidence in the present studies suggest a role for regulation of the SRE by MAPK in GH-stimulated c-*fos* expression. First, GHR that activate MAPK (GHR_{1-638} and GHR_{1-454}) activate c-*fos* expression, whereas GHR that do not activate MAPK (GHR_{1-294}, $GHR_{\Delta P}$) do not activate c-*fos*. Second, the tyrosine kinase inhibitor P11 inhibits GH-stimulated c-*fos* expression under conditions identical to those in which it inhibits tyrosine phosphorylation of ERK1 and -2, but not JAK2 (12, data not shown). Thus, the inhibition of GH-stimulated c-*fos* expression by P11 may reflect the contribution of a P11-sensitive kinase downstream of JAK2 but upstream of MAPK. Third, in cells expressing GHR_{1-638} and GHR_{1-454}, GH stimulated Elk-1-mediated transcriptional activation, whereas cells expressing GHR_{1-294} failed to show such stimulation. Phosphorylation of Elk-1 and subsequent Elk-1-mediated transcription of c-*fos* via the SRE are well characterized events dependent on ERKs (52–55). ERK activation of GH-stimulated Elk-1 transcription is consistent with recent observations that GH stimulates the phosphorylation and activation of Elk-1, facilitating SRE-mediated transcriptional activation of c-*fos* in response to GH (26).

Fourth, the GHR that mediate GH-stimulated c-*fos* expression also mediate stimulation of *egr-1*. The present studies document for the first time that GH stimulates *egr-1* expression, both via endogenous GHR in 3T3-F442A cells and via

expressed GHR in CHO cells. A preliminary report is consistent with GH-stimulated *egr-1* (56). Regulation of *egr-1* by growth factors involves core SRE sequences and Ternary Complex Factor (29, 30, 57), similar to c-*fos*, and would be predicted to be regulated similarly to c-*fos*. In fact, expression of *egr-1* and c-*fos* in response to insulin has been reported to involve MAPK activation (58). Whether the mechanism for GH-promoted *egr-1* expression is the same as that for c-*fos* remains to be determined. Inhibition of c-*fos* expression by staurosporine also raises the possibility that PKC could contribute to regulation of c-*fos*, consistent with previous observations (35, 59–61). Possible interactions among these signaling pathways in GH-stimulated c-*fos* or *egr-1* expression remain to be determined.

GHR uses multiple mechanisms to mediate transcriptional regulation in response to GH

As discussed, Elk-1-mediated transcription in response to GH was observed in cells expressing only the N-terminal half of the cytoplasmic domain of GHR (GHR_{1-454}). Consistent with this, stimulation of SRE-mediated luciferase expression by GH (26) was evident in CHO cells expressing GHR_{1-638} or GHR_{1-454}, but not GHR_{1-294} (data not shown). These observations emphasize the difference between SRE-mediated *trans*-activation of c-*fos* and Stat5-mediated *trans*-activation of the *spi-2.1* gene. GHR_{1-454} does not mediate activation of *spi-2.1* by GH (3, 4, 7, 45). The promoter of *spi-2.1* contains GLEs that bind tyrosyl-phosphorylated Stat5 in response to GH (2). Stat5-dependent transcriptional activation via the GLE requires both box 1 and the C-terminal half of the cytoplasmic domain of GHR (4). Activation of Stat5 and *spi-2.1* transcription thus appear to require JAK2 as well as a second GH-dependent signaling event involving C-terminal sequences in GHR, possibly the one mediating GH-stimulated calcium oscillations (4).

The c-*fos* promoter contains sequences other than the SRE, including the Sis-inducible element (SIE), an Activating Protein-1 site, and a Calcium/cAMP Response Element, all of which are required for expression of c-*fos in vivo* (62). The SIE binds Stat1 and -3 (20–22, 63). Presumably mediated by a JAK2-dependent pathway, Stat1 and -3 are tyrosyl phosphorylated and bind to the SIE in response to GH. The SIE can mediate GH-stimulated reporter expression in cells overexpressing Stat3 (9). The activation and binding of Stats 1 and -3 are reported to occur in cells expressing only the N-terminal half of the cytoplasmic domain (GHR_{1-454}) (8, 9). Thus, c-*fos* expression in cells expressing GHR_{1-454} may reflect contributions of the SIE as well as the SRE. The relative contributions of SIE and SRE in GH-regulated c-*fos* transcription are currently under study. The present findings argue against a role for the Calcium/cAMP Response Element in GH-stimulated c-*fos* expression based on the requirement for the C-terminal half of the cytoplasmic domain for GH-stimulated calcium oscillations (4), but not for GH-stimulated c-*fos* expression. This further distinguishes regulation of c-*fos* and *spi-2.1* by GH, supporting the idea that the mechanisms by which GHR mediate c-*fos* and *spi-2.1* expression differ and suggesting two distinct signaling mechanisms for GHR-mediated induction of c-*fos* and *spi-2.1*.

Multiple signaling pathways may mediate GH-stimulated glucose uptake

The ability of GH to stimulate glucose uptake was found to require box 1 of GHR, did not require the C-terminal half of the cytoplasmic domain of GHR, and was blocked by tyrosine kinase inhibitors that inhibit JAK2 as well as an inhibitor that does not. The requirement for box 1 is consistent with a role for association of GHR and JAK2 in GH-stimulated glucose uptake. One JAK2-dependent pathway by which GH might regulate glucose transport that is consistent with these results involves IRS-1 and -2. The current view of insulin-stimulated glucose transport involves insulin receptor-mediated tyrosyl phosphorylation of IRS-1 and -2, leading to association of IRS-1 or -2 with phosphatidylinositol-3′ kinase (PI-3K) and activation of PI-3K, which is required for recruitment of Glut4 glucose transporters to the plasma membrane (64). GH, like insulin, stimulates the tyrosyl phosphorylation of IRS-1 and IRS-2, presumably via JAK2 (17, 65). Like stimulation of glucose transport, the ability of GHR to transduce the signal for IRS-1 and IRS-2 tyrosyl phosphorylation in response to GH requires box 1, but does not require the C-terminal half of the cytoplasmic domain of GHR (17). In response to GH, phosphorylated tyrosines in IRS-1 and IRS-2 bind and activate PI-3K (17, 65). The finding that wortmannin, an inhibitor of PI-3K, inhibits GH-stimulated lipogenesis (66) supports a role, either direct or indirect, for PI-3K in the regulation of glucose transport by GH. Further, GH, like insulin, rapidly stimulates glucose transport in adipocytes by recruiting Glut1 and Glut4 glucose transporters to the plasma membrane (67, 68).[1] Taken together, the available data support a JAK2-dependent mechanism for GH-stimulated glucose uptake potentially mediated via IRS-1 or -2 and PI-3K.

A MAPK-mediated pathway may also contribute to the events by which GHR mediate stimulation of glucose uptake. P11 blocks GH-induced tyrosyl phosphorylation of ERK1 and -2 but not JAK2 (12, data not shown). The partial inhibition of glucose transport by P11 may reflect the contribution of one or more P11-sensitive kinases downstream of JAK2 but upstream of MAPKs, as discussed for c-*fos* expression. A possible role for MAPK in regulation of glucose transport is supported by observations that expression of Raf-1, a component of MAPK-mediated pathways, increases glucose uptake in 3T3-L1 cells (69). Activation of MAPK in GH-treated cells may be modulated by PI-3K, as MAPK activation is blocked by the PI-3K inhibitor wortmannin (70)[2], which also interferes with translocation of MAPK to the nucleus (71). The present data are thus consistent with involvement of JAK2 and another downstream tyrosine kinase, such as ERK1 or -2, or proteins regulating IRS-1, IRS-2, or PI-3K in the stimulation of glucose uptake by GH.

The inhibition of GH-stimulated glucose uptake by staurosporine observed in 3T3-F442A fibroblasts in the present study is in agreement with the inhibition by staurosporine reported for GH-stimulated lipogenesis in rat adipocytes (66). The concentration of staurosporine (500 nM) used in the present experiments blocks GH-promoted tyrosyl phosphor-

[2] Hodge, C. L., and J. Schwartz, unpublished observations.

ylation of virtually all GH-responsive cellular proteins (12, 66, data not shown). Herbimycin A, which inhibits tyrosine kinases by a mechanism different from that of staurosporine, inhibited glucose uptake as effectively as staurosporine in the present study. The failure of herbimycin A to interfere with GH responses, including lipogenesis and JAK2 phosphorylation, reported in rat adipocytes (66) most likely reflects the short exposure time (10 min) to the inhibitor relative to the 18- to 22-h exposure used in the present studies. As staurosporine is also an inhibitor of PKC, which has been implicated in GH-induced lipid metabolism in rat adipocytes (72–74), a role for PKC in GH-stimulated glucose uptake can also be considered. Nevertheless, although each inhibitor cannot be stated to be absolutely specific for individual tyrosine kinases, the present observation that GH-stimulated glucose uptake was inhibited by all three of the diverse tyrosine kinase inhibitors used supports the general conclusion that tyrosine kinases participate in GH-stimulated glucose uptake. Such kinases appear to include a kinase(s) downstream of JAK2 in addition to JAK2.

In summary, responses to GH implicated in growth promotion (early response gene expression) and metabolism (glucose uptake) require association of JAK2 and GHR, distinguishing them from GH-induced calcium oscillations, which are thought to be independent of JAK2. Further, the mechanism for SRE-mediated induction of c-*fos* transduced by the N-terminal half of the cytoplasmic domain of GHR is distinct from that for Stat5-mediated transcription of *spi-2.1*, indicating that GH uses multiple mechanisms to signal to the nucleus.

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