Signalling Pathway of GH

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Abstract. GH has long been known as a regulator of body growth and metabolism, yet its mechanism of action at the cellular level has been elusive. We have recently shown that GH promotes the rapid association of GH receptor with the tyrosine kinase JAK2, activates JAK2, and promotes the tyrosyl phosphorylation of both JAK2 and GH receptor. This suggests that the initial signalling event in GH action is the activation of JAK2 which in turn phosphorylates tyrosines within JAK2 and GH receptor. We have identified a number of proteins that appear to bind to these phosphotyrosines in GH receptor/JAK2 complexes. These proteins in turn become phosphorylated on tyrosines, resulting in their activation. These proteins include: 1) the signal transducers and activators of transcriptions (Stats) 1, 3 and 5 which have been implicated as regulators of transcription of a variety of genes; 2) the insulin receptor substrates (IRS) 1 and 2, which are believed to mediate some of the metabolic effects of GH; and 3) Shc proteins which lie upstream of Ras and the mitogen activator kinases (MAP) designated ERKs 1 and 2, proteins implicated in the regulation of cellular growth and/or differentiation. These various proteins work in concert with each other and with other signalling molecules to elicit the diverse effects of GH. Other hormones and growth factors also activate JAK kinases. Specificity in signalling was investigated by determining whether signalling pathways for particular ligands may be selectively inhibited by hormones or growth factors. Glucocorticoids were found to selectively decrease binding and cellular signalling in response to GH. This decrease appeared to be due to a decrease in the number of GH receptors in the plasma membrane. Using truncated and mutated GHR, two regions of the GH receptor were identified required for the inhibitory effect of glucocorticoids. Interestingly, they appeared to differ from the region required for GH-induced internalization. Hence, a large amount of insight into signalling by GH has been obtained during the 3 years since JAK2 was identified as a signalling molecule for GH and other ligands that bind to members of the cytokine receptor family. This new insight, and the insight that will continue to be gained in the next few years should enable the design of new and better therapeutic uses of GH and the other ligands that bind to JAK kinase-linked receptors.

Key words: GH, JAK2, MAP kinases, Insulin receptor substrate-1 (IRS-1), Signal transducers and activators of transcriptions (Stat)

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GH is well known for its effects on body growth and metabolism. Yet the cellular and molecular mechanisms by which GH elicits its physiological effects remained elusive for many years. Our laboratory hypothesized that GH may act like a number of other growth factors (e.g. insulin-like growth factor, epidermal growth factor (EGF),

platelet-derived growth factor) to activate a cellular tyrosine kinase. Either the GH receptor was itself a ligand-activated tyrosine kinase or was associated with a tyrosine kinase that became activated upon ligand binding [1–3]. Four years ago, we were able to provide evidence that the GH receptor itself is not a tyrosine kinase, but rather associates with a tyrosine kinase of size similar to the GH receptor (~120–130 kDa) [4, 5]. We then identified JAK2 as the tyrosine kinase that is acti-

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Fig. 1. GH receptor signaling pathways. Possible signaling pathways initiated by binding of GH to its receptor are shown. The shaded molecules and solid arrows indicate molecules and pathways regulated by GH. The dotted arrows and unshaded molecules indicate molecules or pathways utilized by other growth factors but not yet known to be involved in GH-dependent signal transduction, or pathways with only limited data to support their existence.

vated in response to GH [6] (Fig. 1). JAK2 is a member of the Janus kinase family of tyrosine kinases, presently consisting of JAK1, JAK2, JAK3 and tyk2 [reviewed in 7]. These kinases are characterized by the presence of a kinase-like domain, in addition to a kinase domain. They have a number of characteristics that made them candidates for the GH receptor-associated kinase. They were of the appropriate size (~130 kDa) to be the GH receptor-associated tyrosine kinase. By amino acid sequence analysis, they were predicted to contain no transmembrane domain. Further, tyk2 had recently been identified as being a signalling molecule for interferon- α (IFN α), whose receptor is distantly related to the GH receptor. In these initial studies, GH was shown both to promote JAK2 binding to GH receptor and to activate JAK2 tyrosine kinase activity [6]. GH was also shown to promote tyrosyl phosphorylation of both JAK2 and GH receptor. These effects were observed within 30 sec of GH addition to the cells, and at low physiological concentrations of GH, suggesting that

activation of JAK2 may be the initial step in GH signal transduction.

JAK family members are now known to be activated by most, if not all, members of the cytokine superfamily of receptors [reviewed in 7]. This family includes the receptors for erythropoietin, interleukin (IL) 2-7, 9-15, IFN γ , IFN α , leukemia inhibitory factor (LIF), Oncostatin M, granulocyte-macrophage colony stimulating factor, granulocyte colony stimulating factor, thrombopoietin, and ciliary neurotrophic factor. These receptors were originally classified together as a family based upon limited homology in their extracellular domain (4 cysteine residues and a WXSWS motif) [8, 9]. More recently, it was recognized that many contain a proline-rich motif (box 1) which, for many of the receptors, has been implicated in JAK binding [10, 11].

The observation that JAK2 is a signalling molecule for GH as well as for other members of the cytokine receptor family suggests that members of the cytokine receptor family may share at least

some of the same downstream signalling pathways. This observation led us to examine whether the signal transducers and activators of transcriptions (Stat) transcription factors, originally identified as transcription factors involved in IFN signalling pathways, might serve as signalling molecules for GH. Multiple Stat proteins have recently been cloned. They are latent cytoplasmic transcription factors that require tyrosyl phosphorylation for activation [reviewed in 12]. Based upon a large body of evidence, it has been hypothesized that ligand binding to a cytokine receptor family member stimulates the activity of the receptor-bound JAK family member which in turn phosphorylate tyrosines in the receptor. These phosphorylated tyrosines can then bind the Src homology-2 (SH2) domain of specific Stat proteins [13, 14]. The Stat proteins are then phosphorylated on tyrosines (presumably by the JAK kinase), homo or heterodimerize, move to the nucleus, bind to specific DNA sequences in the promoter regions of various genes, and regulate gene transcription.

In the case of GH, we and others examined whether GH activates Stats 1, 2, 3 and 5. In 3T3-F442A fibroblasts, CHO and COS cells expressing cloned rat liver GH receptor, and livers from hypophysectomized rats, GH was found to stimulate the binding of Stats 1, 3 and 5 [15–20]. No phosphorylation of Stat2 was observed [21]. Consistent with Stats 1, 3 and 5 serving as signalling molecules for GH, GH was also shown to promote the binding of Stats 1, 3 and 5 to DNA. GH was shown to promote the tyrosyl phosphorylation of Stats 1 and 3 to the sis-inducible element (SIE) of the *c-fos* promoter, and Stat5 to the γ -activated sequence (GAS)-like element of the β -casein and Spi 2.1 promoters [16, 19, 20, 22]. The migration of the Stat proteins following GH stimulation is consistent with it being phosphorylated on serines or threonines, as well as tyrosines, raising the question of whether phosphorylation on these amino acids plays a role in the GH activation of Stat proteins. Interesting questions also arise as to how the Stat proteins move from the cytoplasm to the nucleus, and the effect of homo versus heterodimerization on gene transcription.

The observation that multiple ligands activate JAK and Stat proteins raises the issue of where specificity in response arises. One possible source of specificity of response is that each receptor con-

tains tyrosines that reside in a different environment, and that once phosphorylated, the phosphorylated tyrosines within each receptor preferentially bind different Stat proteins. To investigate this hypothesis, we examined regions of the GH receptor that are required for activation of specific Stat proteins [22]. For Stat5, specific regions of GH receptor were implicated, including tyrosines within both the N and C-terminal halves of the receptor. Some Stat5 was activated even in GH receptor in which it was predicted no tyrosines were phosphorylated, raising the possibility that some Stat 5 is activated as a result of binding to JAK2 itself. Similar studies with Stats 1 and 3 suggested that in contrast to Stat 5, activation of these Stats does not require Tyr within the C-terminal of the GH receptor. One or more tyrosines within the N-terminal half of the receptor may be required. Some Stat protein may also be activated as a result of binding to JAK2. Other studies have identified a Stat 3 binding motif that is present in 7 copies in the LIF receptor [13] (and absent in GH receptor), and a Stat 1 binding motif present in the IFN γ receptor [14] (absent in GH receptor). These results suggest that while each ligand that activates a JAK kinase may be capable of activating a small amount of most Stat proteins, particular Stats are preferentially activated by a particular ligand because of the presence of high affinity Stat binding sites in their receptor components.

Since GH is known to be involved in growth and differentiation, it was of interest to determine whether GH activates the MAP kinases, also designated extracellular regulated kinases (ERKs) 1 and 2. MAP kinases are primarily serine/threonine kinases that have been implicated in the regulation of cellular growth and/or differentiation [23]. They have been shown to phosphorylate a variety of cellular proteins, including other kinases, transcription factors, cytoskeletal components, and phospholipases [reviewed in 24]. GH was shown to stimulate rapidly the activity of MAP kinases and at physiological concentrations [25, 26]. ERKs 1 and 2 have been shown to be activated by multiple receptor tyrosine kinases by a pathway involving the binding of Grb2 directly, or via SHC proteins, to phosphorylated tyrosines within the receptor [27]. Grb2 is in a complex with the guanine nucleotide binding protein SOS, which activates Ras guanine nucleotide exchange protein.

Ras then activates the serine/threonine kinase Raf, which in turn activates the threonine/tyrosine kinase MEK, which in turn activates MAP kinases. We examined the early steps in the activation of ERKs 1 and 2 by GH [28]. GH was found to stimulate the tyrosyl phosphorylation of 3 isoforms of SHC protein and the association of Grb2 with Shc, but not with GH receptor or JAK2. These results are consistent with GH activating ERKs 1 and 2 via a Shc-Grb2 pathway. Interestingly, GH appeared not to require specific tyrosines within GH receptor to stimulate the phosphorylation of 2 of the 3 Shc isoforms, suggesting that Shc proteins may interact with JAK2 or an accessory protein, rather than with GH receptor.

Under the appropriate circumstances, GH exhibits insulin-like stimulatory effects on carbohydrate and lipid metabolism [29], suggesting that GH may share signalling pathways with insulin. Since insulin receptor substrate-1 (IRS-1) has been shown to be an important, if not primary, signaling molecule for insulin [30], we examined whether IRS-1 might be a signalling molecule for GH [31]. We showed that like insulin, GH stimulates the rapid tyrosyl phosphorylation of IRS-1 at physiologically relevant concentrations. GH also stimulates the association of the p85 regulatory subunit of phosphatidylinositol phosphate 3' kinase (PI3' kinase) with IRS-1, indicating that IRS-1 does serve as a signalling molecule for GH. In contrast, GH-promoted association of PI3' kinase with JAK2 or GH receptor was not detected. Interestingly, the region of GH receptor required for GH-promoted tyrosyl phosphorylation of IRS-1 was the same as that required for JAK2 binding and activation. No phosphorylated tyrosines within the cytoplasmic domain of GH receptor appeared to be required for IRS-1 phosphorylation. This suggested the possibility that not only is JAK2 required to phosphorylate IRS-1, but also that JAK2, rather than GH receptor, may provide the docking site for IRS-1. Consistent with JAK2 or a protein that binds to GH receptor or JAK2 providing the binding site for IRS-1, other ligands that activate JAK2 (LIF and IFN γ) were also found to stimulate the phosphorylation of IRS-1, roughly in proportion to the ligand's ability to stimulate the tyrosyl phosphorylation of JAK2 (GH>>LIF~IFN γ). Since IRS-1 and PI3' kinase have been implicated in the rapid stimulation by insulin of glucose transport in adipocytes, it is

tempting to speculate that the rapid transient stimulatory effect of GH on glucose transport in adipocytes utilizes an IRS-1-PI3' kinase pathway. In support of this, wortmannin, an inhibitor of PI3' kinase, has been shown to inhibit the ability of GH to stimulate lipid synthesis in rat adipocytes [32]. Glucose transport is believed to be the rate-limiting step for lipid synthesis in these cells.

We further investigated the mechanism by which the many ligands that activate JAK2 kinases may achieve specificity in response. We therefore asked whether other hormones or growth factors might selectively interfere with the ability of particular ligands to activate JAK kinases or downstream signalling events. We focussed our attention on the glucocorticoids, because it is well known that glucocorticoids inhibit growth of children, presumably at least in part by antagonizing the actions of GH in peripheral tissues. Dexamethasone (dex), a potent synthetic glucocorticoid was found to decrease GH-promoted tyrosyl phosphorylation of multiple cellular proteins, including Stat3, ERKs 1 and 2, GH receptor and JAK2. Decreased tyrosyl phosphorylation of ERKs, Stat3 and JAK2 in response to GH was not observed with other activators of one or more of these proteins (e.g. LIF, IFN γ , epidermal growth factor), indicating the response is fairly specific to GH [33]. Further experiments determined that the effect of dex was to selectively decrease the number of GH binding sites in the plasma membrane, indicating that dex alters the distribution of GH receptor in the cell. Consistent with dex not altering the synthesis of GH receptor in the cell, dex was found to decrease the number of GH receptor binding sites in the plasma membrane of CHO cells expressing some truncated GH receptors and not others, even though all cDNA encoding the various GH receptors had the same promoter. Regions of the GH receptor required for the inhibitory effect of dex on GH binding were determined using these truncated and mutated GH receptors expressed in CHO cells. Two regions were identified. One region lies between amino acids 455 and 506. Tyr 333 and/or 338 also appear to be required for the second. Neither of these is implicated in GH-induced internalization of the GH receptor. These results lead to the hypothesis that dex regulates the synthesis of a protein that either binds to these regions, modifies these regions, or interferes with the binding of a protein to these

regions. This modification in turn decreases the rate at which GH receptor enters the plasma membrane or increases the rate at which the GH receptor cycles through the plasma membrane [33].

Taken together, the results described in this paper begin to provide a framework for understanding how GH acts to regulate events in the cell that ultimately result in the observed physiological effects of GH. Thus, GH is believed to bind to its receptor, stimulating association of GH receptor with JAK2 and activation of JAK2. JAK2 then phosphorylates itself and the GH receptor. These phosphorylated tyrosines form binding sites for a variety of signalling molecules or proteins that in turn bind known signalling molecules, including SHC proteins, Stat proteins, and IRS-1. These proteins in turn become phosphorylated on tyrosines, with a resulting activation or capability of binding additional signalling molecules. Although a number of other ligands also activate JAK kinases, specificity in response can be achieved through a variety of means. One example provided here is that other intercellular communicators such as hormones, can regulate specifically the level

of a particular receptor. Another example discussed is that specificity in response can be achieved by the binding of specific signalling molecules to phosphorylated tyrosines present only in certain receptor subunits. Other mechanisms include expression of specific receptors in specific cells or during specific times during cellular differentiation.

Clearly, an enormous amount has been learned about GH signalling in the few years since JAK2 was identified as a primary signalling molecule for GH [6]. Presumably, much more will be learned about the actions of GH, and its interactions with other cytokines and hormones that activate JAK family kinases, in the next several years. This should result in better use of GH and other ligands that activate JAK kinases as therapeutic agents.

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