Mechanism of Growth Hormone Signal Transduction*

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Rapid progress has been made recently in the definition of GH receptor signal transduction pathways. It is now apparent that many cytokines, including GH, share identical or similar signalling components to exert their cellular effects. This review provides a brief discourse on the signal transduction pathways, which have been demonstrated to be utilized by GH. The identification of such pathways provides a basis for understanding the pleiotropic actions of GH. The mechanisms by which the specific cellular effects of GH are achieved remain to be elucidated.

I. INTRODUCTION

The importance of the cloned growth hormone (GH) receptor in the regulation of postnatal somatic growth is evidenced by the demonstration of point mutations and deletions in the gene encoding the GH receptor in Laron type dwarfism[1,2] and sex linked dwarfism in the chicken[3,4]. Furthermore, GH receptor gene deletion in mice results in a growth retarded phenotype[5]. GH acts as autocrine and paracrine growth factor to regulate the proliferation, apoptosis, differentiation, and chemotaxis. The mechanism by which the GH receptor mediates the general pleiotropic and specific somatic responses to its ligand have only recently begun to be understood. This review provides a brief discourse on the signal transduction pathways that have been demonstrated to be utilized by GH. The identification of such pathways, at least, provides a basis for understanding the pleiotropic actions of GH.

II. THE GROWTH HORMONE RECEPTOR

II.1. Cytokine Receptor Superfamily

The GH receptor was the first identified member of the cytokine receptor superfamily[6 8]. Other members of the class 1 cytokine receptor superfamily include prolactin (PRL), erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), ciliary neurotrophic factor (CNTF), thrombopoietin, leptin, interleukins (IL) 2-7, IL-9, IL-11 and IL-12. The superfamily also includes receptor subunits that interact with more than one cytokine receptor. Such receptor subunits include gp130 (shared by LIF, oncostatin M, IL-6, IL11, IL-12), the β-subunit of the leukaemia inhibitory factor (LIF) receptor, the γ-chain of the IL-2 receptor

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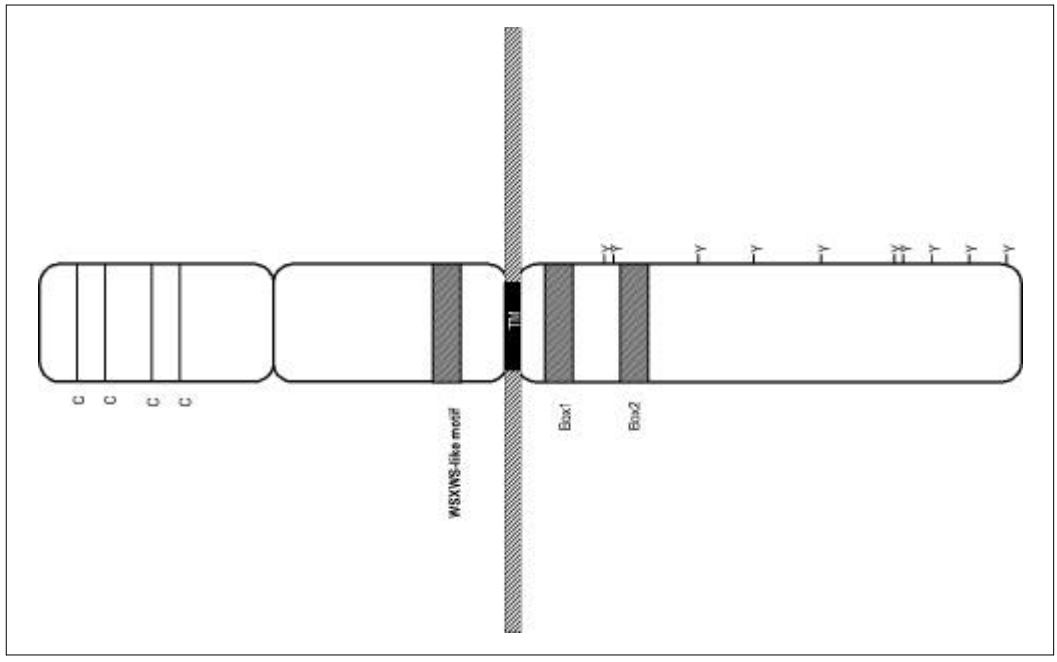

Fig. 1.

(shared by IL-2, IL-4, IL-7, IL-9 and IL-15) and the IL-3 receptor common β chain. A second class of more distantly related cytokine receptors has been classified and includes the α and β subunits of the interferon (IFN)-α/β receptor, both the α and β subunits of the IFN-γ receptor and the IL-10 receptor[7]. Characteristics which group members of the class 1 cytokine receptor superfamily include the following (see Fig. 1)[9,10] (a) possession of a single putative membrane spanning domain; (b) limited amino acid homology (14 44 %) in a region spanning approximately 210 amino acids in the extracellular domain (corresponding to two fibronectin III domains of similar size); (c) conserved pairs of cysteine residues in the extracellular domain and a conserved tryptophan residue adjacent to the second cysteine in the N-terminal fibronectin domain ; (d) a WSXWS (trp,ser,any,trp,ser)-like motif in the C-terminal fibronectin domain (YXXFS in the mammalian GH receptor)[11]; (e) the absence of a canonical tyrosine kinase consensus sequence and (f) two short homologous (proline rich) domains in the intracellular domain. Box 1 is 8 residues in length and is located within 20 residues of the transmembrane domain. Box 1 displays an alYPXalPXP or YXXXalPXP consensus sequence where al is any aliphatic residue, Y is any hydrophobic residue and P is proline. In the mammalian GH receptor the Box 1 sequence is ILPPVPVP[11]. Box 1 is one site of association of Janus Kinase 2 (JAK2) with the GH receptor and is critical for most GH stimulated cellular functions [1 2 15]. Box 2 is less well defined and comprises approximately 15 amino acid residues situated 30 residues distal to Box 1[11]. It consists of a cluster of hydrophobic and acidic residues ending with one or two basic residues. Deletion or mutation of these

boxes abrogates JAK activation and subsequent signal transduction leading to proliferation[16,17]. The growth hormone-binding protein (GHBP) is the soluble circulating form of the GH receptor. GHBP is id to extracellular part of the receptor. It is differentially generated among species: alternative splicing in mice and rat[18], proteolysis of the membrane-bound form of the receptor in other species[19]. Some short but membrane-anchored isoforms of the GH receptor have been described in humans[20,21].

II.2. Receptor Dimerization

With use of recombinant hGH and the extracellular domain of the receptor, the Genentech group has defined the physical interaction between these two molecules[22]. Use of X-ray crystal-logography has enabled the visualization of the three dimensional structure of the receptor extracellular domain (GHBP) and regions that interact with the ligand[23]. One hGH molecule interacts with the extracellular domains of two receptors leading to receptor homodimerization[24]. Accordingly a model was proposed whereby hGH possesses two receptor binding sites; a high affinity site "site 1" and a lower affinity site "site 2" that sequentially interact with binding 'pockets' in two discrete receptor molecules [25]. Contact with hGH at site 2 and a dimerization interface of approximately 500 Å between the two extracellular domains of the receptor stabilize the binding of the second receptor to the complex [22,26]. Interestingly, in the GHBP/receptor the two-ligand binding sites are largely composed of the same amino acid residues and possess similar overall shape and structure. The amino acid residues that form sites one and two in the GHBP are located in six clusters between residues 43 and 218[22].

The resulting receptor dimerization presumably provides the signal to generate the biological response[22,25]. Evidence in support of this hypothesis includes the following; (a) self antagonism for GH receptor activation by GH at high concentration (at high ligand concentration the higher affinity receptor site 1 will be saturated with ligand thereby precluding receptor dimerization via site 2); (b) activation of myeloid cell replication with bivalent but not univalent monoclonal antibodies directed against the GH receptor; (c) disruption of interaction with site 2 by point mutation in the hormone (eg. hGH-G120R) generates GH antagonistic activity *in vitro*[25] and *in vivo*[27] ; (d) Use of a monoclonal antibody that binds to the dimerization interface between the two receptor molecules, and would therefore be expected to prevent dimerization, antagonizes GH dependent cellular proliferation[24] ; (e) naturally occurring receptor isoforms lacking the intracellular domain inhibit the function of the full length receptor presumably by the formation of non-productive dimers[20] and (f) GH independent dimerization of the GH receptor by a leucine zipper results in constitutive activation of STAT5 mediated transcription and cellular proliferation[28]. However, some data suggests that simple dimerization is not sufficient and not all the effects of GH are antagonistic at high GH concentration[29]. Receptor dimerization has similarly been demonstrated to be involved in signal transduction by other cytokine receptors[7,30,31]. With use of such data on dimerization of the receptor, it may be possible to design small molecule agonists or antagonists for the GH receptor, as has been described for the related EPO receptor[32].

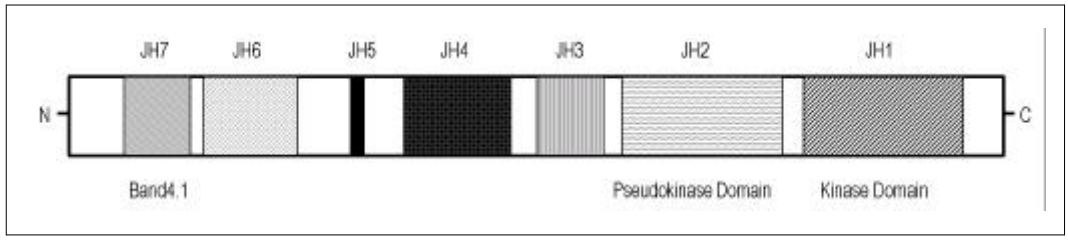

Fig. 2.

III. SIGNAL TRANSDUCTION PATHWAYS UTILIZED BY GH

III.1. Cytoplasmic Tyrosine Kinases

Due to lack of intrinsic kinase activity, members of the cytokine receptor superfamily recruit and/or activate cytoplasmic tyrosine kinases to relay their cellular signals[33]. At least 32 mammalian nonreceptor tyrosine kinases classified into 11 groups have been identified, each with the ability to bind to the intracellular motif of different receptor molecules [34]. They share the similarity on the primary sequence as 1) the Src homology 1 (SH1) domain, also referred to as the catalytic domain. 2) the Src homology 2 (SH2) domain. 3) the Src homology 3 (SH3) domain. Below we review the non-receptor and receptor tyrosine kinases demonstrated to be activated by GH and comment on their possible function in GH signal transduction.

1.1 Janus Kinases

The Janus family of tyrosine kinases are thought to be the predominant non-receptor tyrosine kinases required for the initiation of GH signal transduction upon ligand binding to the receptor[35 37]. Members of the JAK family of tyrosine kinases to date include JAK1, JAK2, JAK3 and Tyk2[8]. They are expressed widely except JAK3, which is found primarily in haematopoietic cells. The unique structural feature of JAK kinase (see Fig. 2) is the absence of SH2 or SH3 domains and presence of 7 conserved JH regions (JH1-JH7), of which the JH1 is a functional catalytic domain and JH2 is a pseudokinase domain. The pseudokinase domain negatively regulates the activity of JAK2, presumably via interaction with the kinase domain and is required to maintain JAK2 inactive in the absence of a stimulus[38]. Interestingly the JH7 domain of JAK contains a domain structurally related to the band 4.1 domain (initially identified in the red blood cell protein band 4.1) and this domain has now been recognized in ERM (ezrin, radixin and moesin) proteins as well as another GH activated kinase, focal adhesion kinase[39,40]. The significance of this domain has not been established but it is postulated that it could bind phosphoinositides [40].

JAKs are constitutively associated with the cytokine receptors and it is likely that ligand binding stabilizes the preformed receptor-JAK complex. Some specificity is evident as the individual cytokine molecules activate different combinations of JAKs and the activation is of both different extent and duration[41]. The predominant JAK utilized by the GH receptor is JAK2[35] although GH has been reported to induce the tyrosine phosphorylation of JAK1[42], JAK3[43] as well. It is not known whether GH also causes tyrosine phosphorylation and activation of Tyk2, although GH dependent phosphorylation of Tyk2 was not detectable in IM-9 lymphocyte[41]. However, the association of the GH

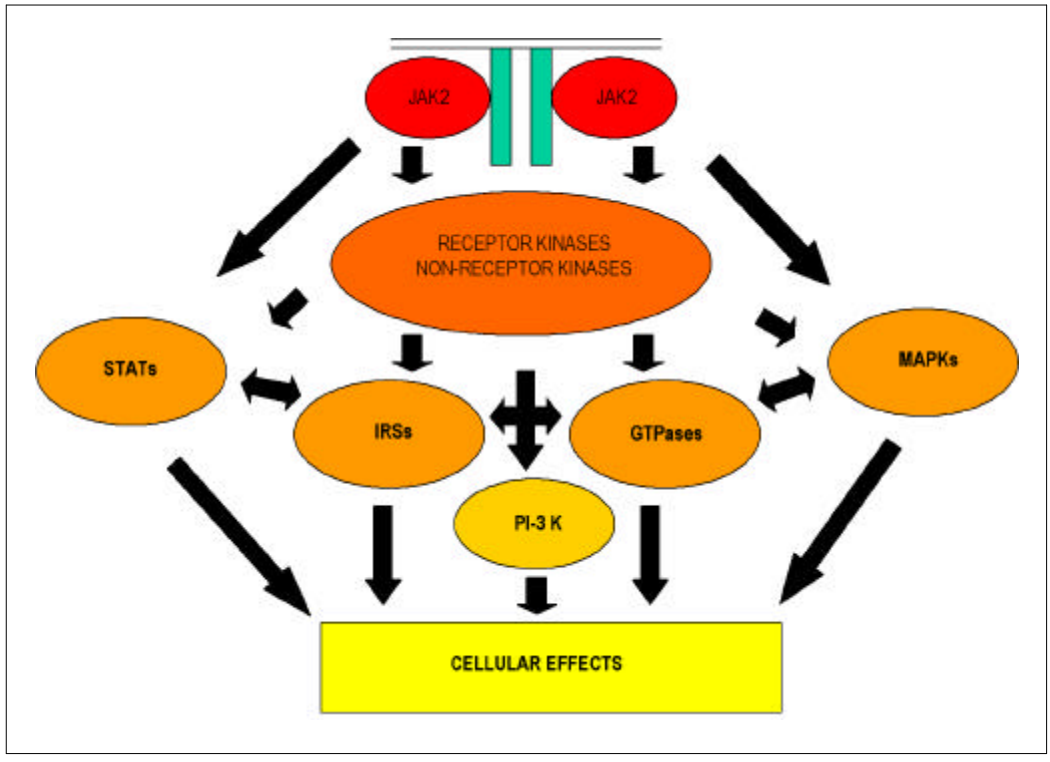

Fig. 3.

receptor with Tyk2 in human liver cells[44] is suggestive that GH may also utilize this kinase for mediation of cellular effects.

Upon GH receptor dimerization (or conformational change as a result of ligand binding to a pre-formed dimer) GH receptor associated JAKs are spatially positioned and/or conformationally modified resulting in trans-phosphorylation and catalytic activation of the kinase domain. The membrane proximal proline rich box 1 region of the GH receptor is required for GH dependent association and activation of JAK2 [14,45]. Although Box 1 is sufficient for activation of JAK2, maximal activation does require more distal residues; apparently required for stabilization of the interaction between the GH receptor and JAK. The box 1 region of the GH receptor is not a canonical proline rich domain and JAKs possess no SH3 domain (which typically bind proline rich domains). It is therefore possible that JAK association with the GH receptor (and other cytokine receptors) could be mediated by an SH3 domain-containing adaptor protein[46]. In this regard it is interesting that we have described the formation of multiprotein signaling complexes after cellular stimulation with GH (see below).

The activated JAKs in turn phosphorylate the intracellular domain of GH receptor, and both the phosphorylated receptor and JAK provide docking sites for a variety of signaling molecules that contain Src-homology 2 (SH2) or other phosphotyrosine binding motifs. The molecules recruited by GH stimulation includes SHC (src homology 2/a collagen-related)[47], IRS proteins[48 50], SHP-1 (SH2-domain-containing protein tyrosine phos-

phatase)[51 53], SHP-2 [51,54], SIRPa [54,55], p125FAK [39,56,57], p85 subunit of PI-3 kinase [58], EGF receptor[59], Grb10[58], Csk (carboxyl-terminal Src kinase)[58], SH2-Bb[54,60,61], APS [62], SOCS-1, -2, -3 and CIS[63 65], PLCg [58,66], and the STAT-1, -3, -5 molecules [42,67 69]. Other JAK2-interacting intracellular signaling proteins that undergo tyrosine phosphorylation upon cytokine/growth factor stimulation, and therefore could be the potential targets downstream of JAK2 in GH signaling include Raf-1[70], Tec[71], STAM (signal-transducing adaptor molecule)[72], STAM2 [73] Vav [74], and Lyn[75]. Signalling molecules which bind directly to the GH receptor are described below (and 58).

To date almost all further downstream signaling pathways utilized by GH apparently require JAK2 activity (Fig. 3). The only reported JAK2 independent effect of GH is Ca^{2+} entry via L-type calcium channels[76]. However it is likely that other uncharacterized pathways stimulated by GH are JAK2 independent including those regulated by cellular Ca^{2+} entry. For example, the related hormone, PRL, has been demonstrated to activate c-Src independent of JAK2[77]. GH has also been demonstrated to regulate Src activity[78] but the JAK2 dependence of its activation has not been investigated. It has also recently been postulated that Src family tyrosine kinase participate in GH signal transduction by mediating Ca^{2+} influx in a JAK2 independent manner[79].

1.2. Src family PTKs

Increasing evidence has accumulated which indicates that other mammalian non-receptor tyrosine kinases may also mediate signal transduction of the cytokine receptor superfamily. It has been demonstrated that Src-kinase family tyrosine kinases can activate and phosphorylate a series of signalling molecules in lieu of JAK kinases[80]. The Src family kinases include Src, Lyn, Fyn, Lck, Hck, Fgr, Blk, and Yes[81]. Most of the Src kinases are widely expressed except Hck, Lck and Blk which display restricted tissue distribution[81].

We have recently demonstrated that GH stimulates the tyrosine phosphorylation and activation of c-Src and c-Fyn[78], both of which exist as components of a multiprotein signalling complex centered around CrkII and p130Cas[78]. Src family kinases serve as a molecular pivot to direct multiple extracellular signals through different signalling pathways with resultant diverse cellular events like cell cycle control, cell adhesion and migration, as well as cell proliferation and differentiation[81]. The function of activated c-Src and c-Fyn in GH signal transduction are still undefined. However, several of the proteins that we have described in the GH stimulated signaling complex have been reported to be substrates for c-Src. These include FAK, p130Cas [82] and c-Cbl[83]. The formation of a FAK-c-Src complex permits c-Src to phosphorylate FAK within the catalytic domain[82] and at tyrosine 925[82] thus enhancing FAK kinase activity and promoting Grb-2 binding, respectively. The binding of Src family PTKs to the motif surrounding the FAK autophosphorylation site (tyrosine 397) is required for Src mediated phosphorylation of tyrosine 925 in vivo [82]. Overexpression of the c-Src binding site mutant of FAK (Phe 397) inhibits fibronectin stimulated signaling to p44/42 MAP kinase implying that Src family PTKs may be essential for FAK mediated signaling events[84]. Thus one potential pathway for the activation of p44/42 MAP kinases by GH is through a c-Src-FAK-Grb-2 complex (see below). c-Src has also been demonstrated to be required for the tyrosine phosphorylation of p130Cas and the localization of p130Cas to the focal adhesion complex [85,86]. It has also been postulated that c-Src bound through its SH2 domain to phosphorylated FAK

facilitates c-Src SH3 domain interactions with p130Cas thereby promoting the formation of a ternary complex of c-Src, FAK and p130Cas. Thus c-Src may be critical for the formation of the multiprotein signaling complex stimulated by GH (see below).

It is likely that GH may utilize Src kinases for functions similar to that reported for PRL and other cytokine receptor ligands. PRL has been demonstrated to stimulate the association of the PRL receptor with c-Src[87] and c-Fyn[88] with resultant activation of tyrosine kinase activity. It has been postulated that c-Src is involved in PRL stimulated mitogenesis of hepatocytes[87]. c-Fyn is utilized by PRL for activation of phosphoinositide-3 (PI-3) kinase. Thus, the association of the p85 subunit of PI-3 kinase with c-Fyn results in PRL stimulated activation of PI-3 kinase[89,90]. c-Fyn is presumed to phosphorylate c-Cbl at tyrosine 731, a binding site for PI-3 kinase[91]. c-Src has also been demonstrated to play a critical role in the erythropoietin (EPO)-induced proliferation and differentiation of K562 cells[92] as well as IL-3 stimulated cellular proliferation[87,93]. Activated Src kinases have also been demonstrated to directly associate with and activate STATs 1, 3, and 5[75] and therefore GH activation of Src family PTKs may regulate STAT mediated transcriptional events stimulated by GH. However, overexpression of either wild type or dominant negative Src in BRL cells stably transfected with GH receptor cDNA does not affect GH stimulated STAT5 mediated transcription (Graichen and Lobie, unpublished)

Interestingly, the major negative regulator of Src kinases, carboxyl-terminal Src kinase (Csk) has also recently been implicated in GH signal transduction by its direct association with the GH receptor[58]. Further investigation is required to elucidate the functional role of Src family PTKs in GH signal transduction.

1.3 Focal Adhesion Kinase (FAK)

Focal adhesion kinase (p125FAK) was originally identified as an abundantly tyrosyl-phosphorylated protein in v-Src-transformed cells[85,94,95]. Localization of p125FAK by immunofluorescence suggests that it is primarily found in cellular focal adhesions, leading to its designation as focal adhesion kinase (FAK)[85,95]. FAK and another FAK-related homologous protein designated proline-rich tyrosine kinase (Pky2) define a subfamily of nonreceptor PTKs. p125FAK associates with integrins in focal contact sites and clustering of integrins via binding to their extracellular matrix ligands leads to activation of FAK. Thus p125FAK has been postulated to play a central role in the response of the cell to the ECM [85,95]. FAK has also been demonstrated to be utilized by non-integrin stimuli to mediated several cellular responses such as alteration in cell morphology and motility and cellular proliferation [85,95,96]. Among the diverse actions of GH is the stimulation of chemotaxis and migration of monocytic cells[97]. Concordantly, we have demonstrated that GH stimulates the re-organization of the actin cytoskeleton in cells with fibroblastic morphology[98]. GH initially stimulates depolymerization of actin resulting in stress fibre breakdown followed by repolymerization and the formation of membrane ruffles[98]. We have demonstrated that GH activates focal adhesion kinase (FAK) and this activation results in the tyrosine phosphorylation of two FAK associated substrates, namely paxillin and tensin[39]. GH stimulation of FAK and presumed subsequent changes in the organization of the actin cytoskeleton has also been demonstrated by other investigators in diverse cell types[56,57]. FAK and paxillin are constitutively associated in the unstimulated state, remain associated during the stimulation phase and recruit tyrosine phosphorylated tensin to the complex after

GH stimulation. Half of the carboxylterminal region of the GH receptor is dispensable for FAK activation but FAK activation does require the proline rich box 1 region of the GH receptor indicative that FAK is downstream of JAK2[39]. FAK associates with JAK2 but not JAK1 after cellular stimulation with GH[39] and use of the JAK2 specific inhibitor, AG490, prevents the GH stimulated tyrosine phosphorylation of FAK[56]. The use of FAK by cytokine receptors provides a possible mechanism for the interaction of cytokine signaling pathways and those utilized by the ECM. By use of FAK replete and FAK deficient cells we have demonstrated that FAK is not required for GH stimulated STAT5 mediated transcription, indicative at least that FAK is not the point of convergence of the cytokine receptor and ECM STAT mediated transcription. It is interesting to note that another group has demonstrated that Pyk2 is a downstream mediator of the IL-2 receptor-coupled JAK3 and STAT5 activation is not affected by dominant negative Pyk2[99]. However, the observed JAK3-dependent activation of Pyk2 is essential for JAK mediated p44/42 MAP kinase and Stat1 activation by IFN-γ[100].

The use of FAK by GH for signal transduction permits the GH signal to be propagated through multiple alternate transduction pathways. Tyrosine phosphorylation of the p85 subunit of PI-3 kinase is regulated by cell adhesion in vivo and it can be phosphorylated by FAK in vitro[101] suggestive that PI-3 kinase may be a substrate of FAK in vivo. The association of FAK and PI-3 kinase is direct and dependent on FAK autophoshorylation[101,102]. It is therefore possible that GH may utilize the FAK-PI-3 kinase pathway to increase phosphatidylinositol 3,4,5-triphosphate levels within the cell in addition to the IRS-PI-3 kinase pathway (see below). It was previously postulated that GH utilized the insulin receptor substrates for activation of PI-3 kinase [49,103,104]. Although we observe some PI-3 kinase activity to be associated with IRS-1, the majority of total cellular PI-3 kinase was not associated with IRS-1 (see below) and by use of IRS-1 deficient cells we have demonstrated that IRS-1 is not required for GH stimulation of PI-3 kinase activity (Goh and Lobie, in preparation). Such potential utilization of two alternate pathways to activate the same kinase may permit the use of PI-3 kinase for distinct cellular purposes. For example, the IRS-1 associated PI-3 kinase may be involved in GH stimulation of metabolic events such as lipogenesis [105] whereas activation of PI-3 kinase via FAK may regulate GH stimulated cytoskeletal re-organization[98]. The alternate use of pathways would allow the cell to respond precisely to hormonal stimuli dependent on cell type and differentiation status. GH has also been demonstrated to stimulate the activation of p44/42 MAP kinase via JAK2 associated SHC and Grb2[14,106]. Tyr-925 of FAK is phosphorylated by c-Src and serves as a binding site for the Grb2-Sos complex both in vivo and in vitro[102]. In addition, the SHC adaptor protein can also bind to the Tyr-397 site in FAK via its SH2 domain. Since FAK, SHC and Grb2[102] associate with JAK2, it is possible that the association of SHC and Grb2 to JAK2 is mediated by FAK and that FAK is an upstream intermediary in the GH stimulation of the p44/42 MAP kinase pathway[102]. It has also been recently reported that the FAK related kinase Pyk2 is required for the activation of p38 MAP kinase[107]. p38 MAP kinase is also activated by GH (108, see below). We have recently reported the activation of JNK by GH via a multiprotein complex encompassing FAK associated CrkII-C3G (Rap1-GEF)[78]. An alternate pathway may also use activated FAK to stimulate JNK through a novel pathway involving the recruitment of paxillin to the plasma membrane and the

subsequent activation of small GTP-binding proteins of the Rho family[109]. p125 FAK may therefore be a JAK2 dependent mediator of many of the pleiotropic actions of GH.

III.2. Receptor tyrosine kinases

The members of cytokine receptor superfamily are generally believed to elicit their signaling by utilizing non-receptor tyrosine kinases such as JAK and/or Src family kinases (see above). Receptor tyrosine kinases (RTKs), to date including 20 functionally related subfamily members[110], are generally thought to function independently, with homo-or hetero- dimerization occurring only within the same subfamily. However, recent reports[111,59] have suggested cross-talk at the receptor level between members of cytokine receptor superfamily and RTKs or between RTKs through direct or indirect interaction with each other at the surface of the same cell. For example, the Kit RTK, which is activated by the hematopoietic stem cell factor, can bind and phosphorylate the cytoplasmic region of the erythropoietin receptor[111].

GH has been demonstrated to functionally utilize members of the epidermal growth factor receptor (EGFR) subfamily. The subfamily consists of four closely related receptors: the EGFR (ErbB1), ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4). EGFR has been demonstrated to be tyrosine phosphorylated but not catalytically activated by JAK2 upon GH stimulation[59], unlike G-protein-coupled receptor mediated EGFR transactivation, where intrinsic EGFR activity is required for downstream signaling[110]. GH stimulated phosphorylation of EGFR serves primarily as a scaffold using Tyr1068 (the major Grb2 binding site) to further elicit the activation of p44/42 MAP kinase [59]. GH has also been reported to stimulate the tyrosine de-phosphorylation and serine/threonine phosphorylation of ErbB2[112] in 3T3-F442A preadipocytes where cellular treatment with GH results in cell cycle arrest[113]. The GH induced decrease in ErbB-2 tyrosine phosphorylation correlated with the attenuation of EGF induced mitogenesis. However, our unpublished data has demonstrated GH stimulated tyrosine phosphorylation of both ErbB2 and PDGFRb in NIH-3T3 and CHO-GHR cells (Zhu and Lobie, unpublished). In this regard it is interesting to note that a recent investigation[114] has demonstrated autocrine secretion of prolactin PRL resulted in the constitutive tyrosine phosphorylation of ErbB2 by JAK2 with resultant Grb2 coupling and activation of Ras-p44/42 MAPK in human breast cancer cell lines. As a result, the human breast carcinoma samples with enhanced ErbB2 expression have higher proliferative and metastatic activity in the presence of autocrine secretion of prolactin[114]. The engagement of RTKs in GH signal transduction, and the resulting alteration in RTK tyrosine phosphorylation, may therefore relate to the cellular requirement for proliferation. In any case. RTKs will presumably be central to mediating many of the pleiotropic effects of GH.

III.3. Multiprotein Complex

The assembly of multiprotein signalling complexes is a common mechanism of signal transduction for various receptors[115]. Multiple signal transduction pathways parade through oligomerization of proteins into multi-subunit complexes, which include different enzymes and adapter molecules. Although adaptor molecules possess no intrinsic enzymatic activity, their ability to mediate protein-protein interactions is vital for the integration and propagation of the signal transduction cascade. Most of these adaptor proteins in GH signal transduction identified to date are SH2/SH3 domain containing proteins or PTB

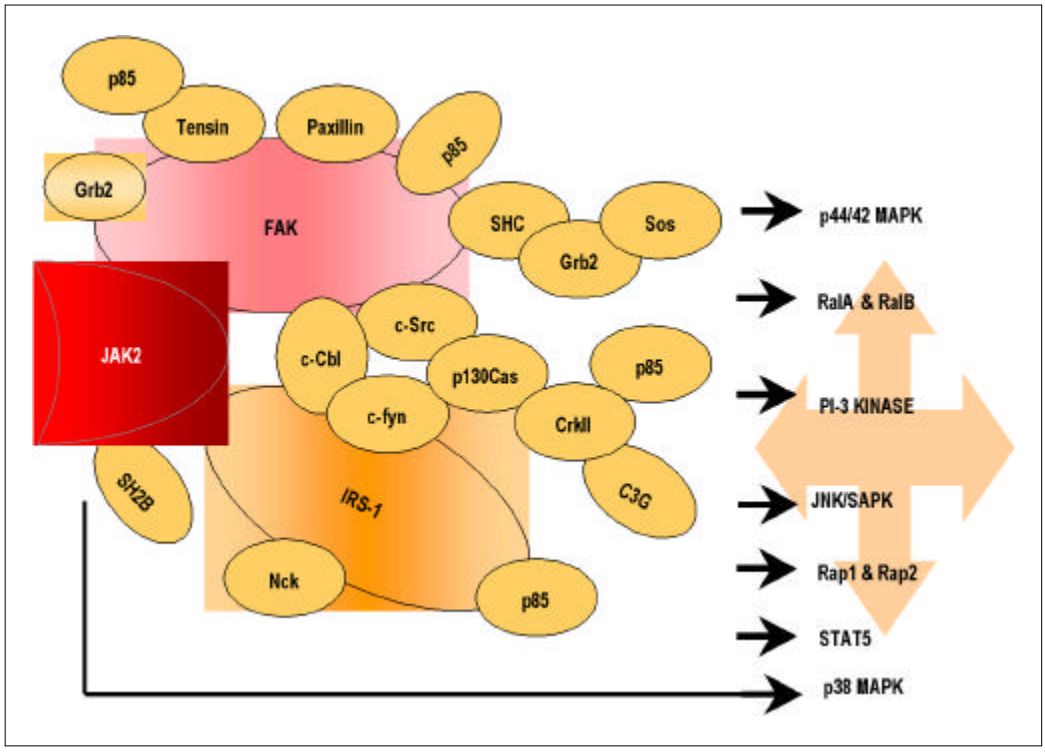

Fig. 4.

(phosphotyrosine binding) domain containing proteins. The major function of these adaptors, such as Grb2, Nck, and Crk, is to recruit proline-rich effector molecules to tyrosine-phosphorylated kinases or their substrates. We have described that GH stimulates the formation of a multiprotein signaling complex centered around CrkII and p130 Cas. A diagrammatic representation of the complex is presented in fig 4. We will describe some of the individual molecules contained in this complex and examine their function or potential function in GH signal transduction (see below). It should be noted that other groups have described the formation of other smaller multiprotein signaling complexes after cellular stimulation with GH including one containing the GH receptor and JAK2, SHP-2 and a glycoprotein with properties consistent with being a SIRP-alpha-like molecule[54] ; and another containing STAT5, p42 MAP kinase, shc and a 63 kDa serine phosphorylated protein resembling Akt-1 [116].

3.1. IRS-1, IRS-2, IRS-3 and PI-3 kinase

GH and insulin share many cellular effects in common including the stimulation of amino acid transport, protein synthesis, glucose transport, lipogenesis, gene expression[117], mitogenesis, prevention of apoptosis[118], differentiation, and re-organization of cytoskeletal architecture[98]. Thus, GH and insulin can be expected to utilize common components of signal transduction pathways. Consistent with this is the ability of GH to stimulate the tyrosine phosphorylation of insulin receptor substrate-1, -2 and -3 (IRS-1, -2, -3)[48 49,103 104,119]. Whether GH also stimulates the tyrosine

phosphorylation of IRS-4[120] has not been investigated. Other cytokine molecules have also been demonstrated to stimulate the tyrosine phosphorylation of IRS-1, IRS-2, IRS-3 (including IL-2, IL-4, IL-7, IL-9, IL-13, IL-15, LIF, OSM, IFN-g and IFN-a) indicative that the IRS molecules may also be a common component of the cytokine receptor superfamily signal transduction pathway [121]. IRS proteins bind directly to several enzymes and adaptor proteins and many of these interactions require tyrosine phosphorylation of IRS proteins. These IRS associated molecules include the p85 regulatory subunit of PI3-kinase, SHP-2, Fyn, Grb-2, Nck, CrkII, CrkL, Csk[121,122] and the Ca2+-ATPases SERCA1 and SERCA2[123]. GH has been demonstrated to activate or tyrosine phosphorylate PI-3 kinase[48,103,105], SHP-2[51, 54], c-Fyn[78], Grb-2[47,124], Nck and CrkII[78]. Indeed, we have demonstrated IRS-1 to be a component of the GH stimulated multiprotein signaling complex centered around CrkII[78]. The GH receptor does not contain the NPXY consensus sequence required for the association of IRS-proteins with receptors such as insulin, IGF-1 and IL-4 [121,122] and no specific tyrosine residues in the GH receptor are required for IRS-1, -2 or -3 phosphorylation[103,104,119]. Although Box 1 of the receptor and therefore presumably JAK2 is required for GH stimulated phosphorylation of IRS-1, -2 and -3[103,104,119] JAK2 also does not possess the NPXY consensus sequence. It is possible that a JAK2 dependent kinase such as FAK (see below) may mediate GH stimulated IRS tyrosine phosphorylation. In any case, the mechanism of the GH stimulated phosphorylation of the IRS proteins will be fundamentally different from the mechanism utilized by insulin[121,122]. It is possible that the IRS proteins may associate with JAK2 via an adaptor molecule and Grb-2 or CrkII may be a suitable candidate[46,47,124]. Interestingly, it has also been demonstrated that FAK associates with, and in conjunction with c-Src, tyrosine phosphorylates IRS-1, concomitant with increased association of IRS-1 with Grb-2, the p85alpha subunit of PI-3 kinase and SHP-2[125]. In addition, FAK-induced association of IRS-1 with PI 3-kinase resulted in increased PI 3-kinase activity but no alteration in p44/42 MAP kinase activity. GH may therefore utilize FAK and associated c-Src to tyrosine phosphorylate at least IRS-1.

PI-3 kinase is a pivotal effector molecule downstream of IRSs and GH stimulated PI-3 kinase activity is associated with either IRS-1 or IRS-2 in a variety of cell types in vitro and in vivo[121,122]. PI-3 kinase phosphorylates inositol lipids at the 3' position of the inositol ring to generate the polyphosphoinositides PtdIns-3-P, PtdIns-3,4-P2, and PtdIns-3,4,5-P3. These lipids then function in signal transduction and membrane trafficking by interaction with 3-phosphoinositide-binding modules in a broad variety of proteins[126]. The newly synthesized PtdIns-3,4,5-P3 is the major polyphosphoinositide required to recruit PDK-1, Akt/PKB, p70S6K, p90RSK, PAK and different PKCs to the plasma membrane, where PDK-1 serves to activate the enzymes by coupling lipids and phosphoryl- groups.

GH has been reported to promote the association of the p85 subunit of PI-3 kinase with IRS-1 [103,104] IRS-2[48,119] and IRS-3[48] and to increase the PI-3 kinase activity associated with IRS-1 and IRS-2[48,103,104,119] and JAK2[127]. There may be some cell type specificity in the mechanism of PI-3 kinase activation by GH as GH stimulation of pancreatic b-islet cells did not result in association of IRS-1 and the p85 regulatory subunit of PI-3 kinase[128] despite IGF-1 stimulation of IRS-1-p85 association. We have also observed a similar phenomenom in NIH-3T3 cells whereby

IGF-1 but not GH stimulates a robust IRS-1-p85 association (Goh and Lobie, in preparation). Also in these cells despite equipotent activation of total cellular PI-3 kinase activity by GH and IGF-1, the majority of GH stimulated PI-3 kinase activity was not associated with IRS-1 (nor IRS-2) in contrast to the situation with IGF-1. Furthermore, GH activation of PI-3 kinase activity was maintained in IRS-1 deficient (IRS-1 -/-) cells in contrast to IGF-1 which required IRS-1 to activate PI-3 kinase (129, Goh and Lobie, in preparation,). Thus, IRS independent pathways may be utilized by GH to activate PI-3 kinase. Indeed, we have demonstrated that CrkII overexpression in NIH-3T3 cells dramatically enhances the ability of GH to stimulate PI-3 kinase activity[130] and this may constitute an IRS independent pathway for activation of PI-3 kinase. It has also been demonstrated that both the p85a and p85b subunit of PI-3 kinase can bind directly to the phosphorylated tyrosine residues at the carboxyl-terminal part of the GH receptor[58] providing a direct alternative route for the activation of PI-3 kinase. Furthermore, a Tec tyrosine kinase has been demonstrated to link JAK1 and JAK2 to PI-3 kinase upon cytokine stimulation (71).

PI-3 kinase is pivotal in many cellular processes including cell proliferation and survival, cytoskeletal re-organization and cellular metabolism[121,122]. Many of the PI-3 kinase dependent events are then mediated by further downstream signaling pathways. GH has been reported to activate in a PI-3 kinase dependent manner the serine/threonine kinase Akt/PKB to deliver an anti-apoptotic signal [131,132]. Several substrates of Akt have been identified including glucose synthase kinase 3 (GSK3), 6-phospho-fructo-kinase (PFK2)[133], GLUT4 and p70S6K. GSK-3 is rapidly phosphorylated at serine 21 in GSK-3α or serine 9 in GSK-3β resulting in inhibition of GSK-3 activity [134]. GH has been demonstrated to inhibit GSK-3 activity in a PI-3 kinase independent manner[135] resulting in GH promoted dephosphorylation of C/EBPb and altered regulation of *c-fos* transcription. Concordantly, GH has also been demonstrated to trigger GLUT4 translocation in a PI-3 kinase dependent manner[136]. Akt has also been linked to the phosphorylation of transcription factors such as c-AMP responsive element-binding protein (CREB) and AP-1, both of which have been demonstrated to be activated by GH[137,138]. GH stimulated p70S6K activation has been demonstrated to be both PI-3 kinase dependent[127,139,140] and PKC-ξ dependent[140]. It is likely that GH stimulated p70S6K activation is mediated through a PI-3 kinase dependent Akt pathway. p70S6K is required for translation of an essential class of mRNAs which contain an oligopyrimidine tract at their transcriptional start (5'TOP), most notably mRNA that are essential for the control of cell proliferation and differentiation[121,127,139,141]. GH also activates PDE4A cyclic AMP-specific phosphodiesterase in 3T3-F442A preadipocytes in a PI-3 kinase-p70S6K dependent manner[139] presumably to allow for the fine regulation of the adipocytic differentiation process. PI-3 kinase has also been reported to partially required for the GH mediated p44/42 MAPK and JNK activation[127,130]. It is apparent that IRS-1 dependent activation of p44/42 MAP kinase is PI-3 kinase dependent (due to the PI-3 kinase dependence of Ras activation) whereas IRS-1 independent activation of p44/42 MAP kinase by GH does not require PI-3 kinase[132]. Furthermore, simple activation of PI-3 kinase does not result in activation of p44/42 MAP kinase as overexpression of CrkII results in PI-3 kinase dependent inhibition of p44/42 MAP kinase despite dramatic enhancement of PI-3 kinase activity[130]. PI-3 kinase has also been shown to be a serine/

threonine kinase that phosphorylates both IRS-1 [142] and STAT3[143] although the biological significance of this activity is not understood. Association of p85 with STAT3 may also constitute an alternate pathway of PI-3 kinase and in accord with the report that STAT3 serves as an adapter to couple PI-3 kinase to the IFNAR1 chain of the type I interferon receptor[143]. We have similarly demonstrated that GH stimulates the association of STAT3 with p85 (Zhu and Lobie, unpublished observations). Other functions of PI-3 kinase include actin cytoskeletal re-organization and we have demonstrated that PI-3 kinase activity is required for GH stimulated actin cytoskeletal re-organization[98]. GH stimulated lipogenesis is also PI-3 kinase dependent[105].

3.2. SHC and Grb2

SHC is a family of SH2 and PTB domain-containing adaptor molecules; the Grb2 molecule consists of one SH2 domain flanked by two SH3 domains. GH was shown to stimulate the JAK2 dependent tyrosine phosphorylation of 66-, 52-, and 46-kDa SHC proteins in 3T3-F442A fibroblasts[47] and the tyrosine phosphorylated SHC proteins associate with JAK2-GHR complexes via its SH2 domain[58]. Tyrosine 469 of the GH receptor is part of a consensus site for SHC binding and the direct interaction of the GH receptor and SHC was observed by the CORT (cloning of receptor targets) technique[58]. When phosphorylated, tyrosine 317 on SHC creates a consensus binding site for Grb2 [144]. In addition to SHC, SHP-2 may be utilized by the GH receptor to recruit Grb2 to a signalling complex[54]. Grb2 can also be recruited to the phosphorylated EGF receptor by either a direct or indirect mechanism upon GH stimulation[59]. This suggests that multiple routes are utilized by GH to mediate membrane translocation of Grb2. The coupling of Grb2-Sos is constitutive and mediated via the interaction of SH3 domain of the Grb2 with C-terminal proline-rich region of the Sos. The SHC-Grb2-Sos pathway is well known to activate p44/42 MAP kinase, but it is reported that different SHC isoforms exert distinct roles in EGF signaling [145,146] (the 66kDa SHC protein is not utilized for activation of p44/42 MAP kinase but results in rapid deactivation of p44/42 MAP kinase). In this regard it is interesting that GH stimulates the association of predominantly the 66kDa SHC with CrkII[78] and expectedly CrkII overexpression results in inhibition of GH stimulated p44/42 MAP kinase activity[130]. A large number of proline-rich proteins known to bind to the SH3 domains of Grb2 have also been reported including c-Cbl, C3G, MEKK1, [147], Shb, SAM68, POB1 and WASP.

3.3. CrkII

CrkII is a member of a family of adaptor proteins (also containing CrkI and CrkL) predominantly composed of SH2 and SH3 domains and has been implicated in various cellular functions such as cytoskeletal reorganization and mitogenesis[148,149]. As described above, GH stimulates the formation of a multiprotein signaling complex centered around CrkII[78]. We have recently demonstrated that CrkII is a powerful positive regulator of GH stimulated PI-3 kinase activity. CrkII enhanced PI-3 kinase activity is used to enhance actin filament re-organization and JNK/SAPK activity and to diminish STAT5 mediated transcription in response to GH[130]. It is presumed that GH activates JNK through a Crk-C3G complex involving the mixed lineage kinase family of proteins[150]. CrkII overexpression also diminishes the GH stimulated p44/42 MAPK activity independent of PI-3 kinase activity[130]. It is expected that the coupling of CrkII-C3G activates Rap1 thereby preventing Raf1 activation by Ras, with resultant lack of MEK activation (151, Zhu unpublished). We have indeed

observed that GH activates Rap1 (Ling, Zhu and Lobie, in preparation). CrkII-p130Cas assembly has also been shown to regulate the Rho and Rac signaling pathway to control cell invasion and survival[148,152]. Activation of Rac by GH has not been reported but Rac has been demonstrated to facilitate the activation of JAK2 by other cytokines[153].

3.4. c-Cbl

c-Cbl comprises an N-terminal transforming region (Cbl-N), which contains a phosphotyrosine binding (PTB) domain, and a C-terminal modular region (Cbl-C) containing a RING finger motif, a large proline-rich region and a leucine zipper. Cbl is a major target of tyrosine phosphorylation in response to stimulation through a wide variety of cell-surface receptors[154]. Cbl is capable of forming multiple signalling complexes with a number of proteins and serves as a negative regulator of tyrosine kinase signalling[147,155]. GH has been demonstrated to induce the tyrosine phosphorylation of c-Cbl and its association with CrkII and p85 subunit of PI-3 kinase[78]. Although it was reported that c-Cbl serves as a RING family E3 that recognizes activated receptor and promotes its ubiquitination by a ubiquitin-conjugating enzyme (E2) and terminates signaling[156], c-Cbl exerts no significant role in the endocytotic internalization of the GH receptor[157]. The role of c-Cbl in GH signal transduction is currently under investigation.

3.5. Grb10

The adapter protein Grb10 belongs to a family of SH2 domain-containing proteins that also include Grb7, Grb14 and the Caenorhabtidis elegans Mig10 [158]. Grb-10 functions in a stimulatory as well as an inhibitory role in mitogenesis depending on the cellular context due to its differential expression in tissue and SH2 binding selectivity[158,159]. Grb10 has been demonstrated to interact with the tyrosine phosphorylated GH receptor[58]. The phosphorylated tyrosines of the region 454-620 of the GH receptor are responsible for this interaction. Grb10 is apparently involved in the regulation of GH stimulated transcriptional events utilizing C/EBP but not STAT5[58].

3.6. SH2-Bβ and APS

SH2-B and APS (adapter protein with a PH and SH2 domain) are the most recent members of a family of tyrosine kinase adapter proteins including Lnk[160]. Both SH2-B and APS are SH2 and PH domain-containing proteins[62,160]. SH2-B includes 3 different splicing variant designated α, β, and γ, [62,160]. SH2-Bβ has been demonstrated to bind to tyrosine phosphorylated JAK2 via its SH2 domain and serve as a substrate of activated JAK2 in GH signal transduction[60]. The tyrosine phosphorylated SH2-Bβ further enhances the GH-stimulated JAK2 kinase activity and subsequent tyrosine phosphorylation of its downstream signalling targets such as STAT3 and STAT5B[61]. SH2-Bβ has also been demonstrated to be required for GH stimulated membrane ruffling and pinocytosis[161] independent of its ability to enhance JAK2 activity. APS and SH2-B have been postulated to play a synergistic role in the regulation of GH stimulated JAK2 activation by the formation of tetrameric complexes that contain two JAK2 molecules to facilitate the association of JAK2 with the activated GH receptor [62].

III.4. Ras-like GTPases

The Ras-like GTPases include H-Ras, K-Ras4A, K-Ras4B, N-Ras, R-ras, Rap1, Rap2, RalA, RalB, TC21, Rheb[162] and M-Ras (R-ras3)[163]. The family is characterized by similarities in the effector domain which Ras utilizes to interact with downstream target molecules. The Ras-like GTPases

play a critical role in multiple signaling pathways leading from various cell-surface receptors[162]. The activation and inactivation of the Ras-like GTPases are controlled by the its conformation change due to a GTP-GDP binding cycle that is controlled by the 2 different regulatory proteins ; 1) Guanine nucleotide exchange factors (GEFs) that promote the formation of active GTP-bound form and 2) GTPase-activating proteins (GAPs) that accelerate the intrinsic GTPase activity of Ras proteins back to the inactive GDP-bound state. Ras and Ras like GTPases are involved in a multitude of cellular effects and many excellent reviews on the structure and function of Ras and Ras like GTPases have been published [162 165].

Ras has been demonstrated to be required for GHR/JAK2-mediated activation of p44/42 MAP kinase[106,166]. As expected from other receptor signaling pathways, GH stimulation of cells results in the assembly of a SHC-Grb2-SOS complex with resultant activation of Ras and subsequent engagement the Raf-MEK pathway[167]. GH stimulated IRS-1 associated PI-3 kinase activity has also been reported to be required for GH stimulated Ras activation[132]. Whether other pathways are utilized for GH stimulated activation of Ras remains to be determined. We have also demonstrated that GH activates both Rap1 and Rap2 (Ling et al., in preparation) and potently activates RalA and RalB (Zhu et al., in preparation). The role of these Ras like GTPases in GH signal transduction is currently under investigation but preliminary results indicate that the Ral molecules are involved in both p44/42 MAP kinase and JAK-STAT mediated transcriptional events.

III.5. MAP kinases

The mitogen-activated protein kinase (MAP kinase) superfamily are proline-directed serine-threonine protein kinases that have important functions as mediators of cellular responses to a variety of extracellular stimuli[168,169]. MAP kinase signalling cascades are evolutionarily conserved and is typically composed of three hierarchical kinases including MAPKK kinase (MAPKKK), MAPK kinase (MAPKK) and MAP kinase (MAPK). To date, more than a dozen mammalian members of the MAP kinase family have been identified[168,169]. Among them the p44/42 MAP kinases (also named extracellular signal-regulated kinases or ERKs), the c-jun N-terminal kinases (also named stress-activated protein kinases or SAPKs) and the p38 MAP kinases have been relatively well characterized. GH has been reported to activate p44/42 MAP kinase[170 172], JNK/SAPK[78] and p38 MAP kinase[108].

The activation of p44/42 MAP kinase is via a well-known sequential cascade involving SHC, Grb2, Son-of -sevenless (Sos), Ras, Raf, and MAP/ERK kinase (MEK)[168,169] and GH has also been demonstrated to utilize this cascade[106,167]. Indeed, Ras and Raf have been demonstrated to be required for GH activation of p44/42 MAP kinase by use of dominant negative forms of H-Ras and Raf-1[106]. As mentioned above there appears to be multiple alternate mechanisms by which GH may initiate this pathway. Interestingly, other signal transduction pathways also regulate the ability of GH to activate p44/42 MAP kinase. PI-3 kinase activity [127,132,157] and protein kinase C (PKC) (specifically PKC-d)[173] are both required for full GH activation of the MAP kinases[127,157]. It is interesting to note that PKC-ξ as a mediator of PI-3 kinase signalling has been shown to activate and phosphorylate Raf-1 and MEK[174]. The activation of p44/42 MAP kinase by GH is also cell type specific[175] indicative that the relative activation of pathways which regulate GH stimulation (or deactivation) of p44/42 MAP kinase may play a

pivotal role in determining the activation state of p44/42 MAP kinase by GH.

p44/42 MAP kinase have been reported to phosphorylate and/or activate further downstream proteins including p70S6K, p90rsk, Sap-1a[183], phospholipase A2(PLA2) [176], c-Raf-1, c-jun, ternary complex factor (p62TCF/Elk1) and STAT molecules[168,169,177 179]. GH has been demonstrated to utilize all of these downstream proteins in its signal transduction pathways[46,180,197]. p90rsk phosphorylates the serum response factor which binds to the serum response element (SRE) of the c-fos promoter[181]. Binding of both SRF and Elk-1 to the SRE contributes to the induction of c-fos gene transcription by GH[166,182]. Elk-1 has been demonstrated to be phosphorylated and transcriptionally activated by GH and both the GH stimulated phosphorylation of Elk-1 and GH stimulated SRE mediated transcriptional activation (and c-fos, egr-1 and jun B expression) were inhibited by the MEK1 inhibitor PD098059[166]. GH has also been reported to utilize Elk-1 to mediate GH-induced transcription of egr-1[183]. Activation of PLA2 by GH increases the level of arachidonic acid and subsequent formation of arachidonic acid metabolites[176]. This GH stimulated PLA2 dependent formation of arachidonic acid metabolites has been implicated in the Ca^{2+} dependent GH stimulation of P4502C12 gene transcription[176]. p44/42 MAP kinase has also been demonstrated to regulate STAT mediated transcription by association with and phosphorylation of STAT molecules on serine residues[184]. Indeed, the MEK1 kinase inhibitor, PD98059, decreases the GH stimulated transcription mediated by STAT5a [185] without affecting the cytoplasmic to nuclear translocation of STAT5a. Phosphorylation by p44/42 MAP kinase can also affect protein stability which has obvious consequences for transcription factor activity[186]. Phosphorylation of FOS by p44/42 MAP kinase and/or p90rsk stabilizes the protein and is required for the transcriptional repression of the c-fos promoter by c-fos[186]. Whether GH also utilizes such mechanisms for transcriptional regulation via the MAP kinase pathway remains to be determined.

We have demonstrated the formation of a multiprotein signaling complex centered around p125FAK[39] and p130Cas-CrkII[78] leading to JNK/SAPK activation. Overexpression of CrkII therefore enhances GH stimulation of JNK/SAPK activity[78,130]. This is concordant with our demonstration that GH stimulation of the cell results in the tyrosine phosphorylation of Nck and the inclusion of both C3G and Nck in the p130Cas-CrkII complex. Both C3G and Nck have been reported to be upstream of JNK/SAPK [187,188]. A CrkII-C3G complex has been demonstrated to activate JNK/SAPK through a pathway involving the mixed lineage kinase family of proteins[150]. Nck connects to the JNK/SAPK pathway by association with SH3 domain associated protein serine/threonine kinases such as PAK or NIK [188,189]. Like p44/42 MAP kinase the activation of JNK/SAPK by GH is also PI-3 kinase dependent [130]. The activation of JNK/SAPK therefore provides another pathway by which GH may affect cellular function. JNK/SAPK is involved in many cellular processes including transcriptional regulation and apoptosis[190] and it is likely that GH utilizes JNK/SAPK for some of these purposes.

We have also recently demonstrated that GH phosphorylates and activates p38 MAP kinase[108] in CHO cells stably transfected with GH receptor cDNA. Again there is apparent cell type specificity as other investigators detected only minimal activation of p38 MAP kinase in 3T3-F442A cells [166]. The activation of p38 MAP kinase by GH is JAK2-dependent[108]. It has recently been reported

that Pyk2 is critical for JAK mediated p38 MAP kinase activation[107] and therefore a JAK2-FAK coupling may be one mechanism for GH activation of the MAP kinase pathway. It has been demonstrated that p38 MAP kinase is required for GH stimulation of ATF-2 and CHOP mediated transcription, for GH stimulated re-organization of the actin cytoskeleton and also for GH stimulated mitogenesis[108]. Interestingly, autocrine production of GH by human mammary carcinoma cells results in the upregulation of the chop gene and subsequent increased CHOP protein expression and GH dependent CHOP mediated transactivation (Mertani et al., submitted). The increased CHOP mediated transcription stimulated by GH is p38 MAP kinase dependent and utilized to prevent apoptotic cell death resulting in increased mammary carcinoma cell number. Thus it is apparent that GH activation of p38 MAP kinase is pivotal in mediation of the pleiotropic cellular effects of GH.

III.6. Phospholipase C/Protein Kinase C/Ca2+ Pathway

A ubiquitous signaling mechanism for members of the cytokine receptor superfamily is use of phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylinositol 4, 5-bisphosphate to produce the metabolite second messenger molecules inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). The former provokes a transient increase in intracellular free Ca2+, while the latter serves as a major direct activator of protein kinase C, although Ca2+ can also induce the activation of PKCs by increasing the affinity of PKCs for anionic phospholipid[191,192]. GH has been demonstrated to increase PLC activity in proximal tubular basolateral membranes from canine kidney[193]. PLC-g was also demonstrated to be tyrosine phosphorylated by GH via its direct binding to JAK2/GH receptor complexes[66]. A tyrosine phosphorylated intracellular portion of the GH receptor has also been demonstrated to bind to PLC-g[58]. GH has also been demonstrated to elicit rapid but transient increases in cellular DAG levels through the utilization of variant pathways in different cells [194 196]. The GH dependent DAG increases in renal proximal tubule cells is accompanied by a rapid transient increase in IP3 suggestive of GH stimulated DAG production from phosphatidylinositol[193]. In contrast, in Ob1771 preadipocytes, GH stimulates the formation of DAG associated with phospholipase C mediated hydrolysis of phosphatidylcholine[195]. GH induced DAG formation is inhibited by a tyrosine kinase inhibitor, which also inhibits JAK2, suggestive that PLC is downstream of JAK2[197]. It worthwhile to note that GH treatment does not always result in the concomitant elevation of intracellular IP3 and/or DAG. This process seems to be cell type specific[46]. For example, GH has been reported to increase DAG content without an increase in IP3 in b-islet cells[198].

The majority of PKC isoforms require DAG for their activation and translocation from the cytosol to the plasma membrane and therefore PKC is required to couple and propogate GH signal transduction from PLC[191,192]. PKC comprises at least 12 structurally related phospho-lipid-dependent kinases and its activity is controlled by autophosphorylation and transphosphorylation, both of which are believed to be mediated by phosphoinositide-dependent protein kinases (PDKs) and tyrosine kinases. The activated PKC is associated with a wide range of interacting proteins and therefore serine/threonine phosphorylates a variety of cellular proteins[199]. Several GH stimulated cellular events are reduced by depleting PKC activity with chronic treatment of phorbol 12-myristate 13 acetate or by PKC inhibitors indicative that PKC is required for cellular functions

such as lipogenesis[200], the expression of the c-fos gene[194,200,201], the increase in intracellular Ca^{2+} concentration[202], the ability of nuclear proteins to bind to the C/EBP consensus sequence[203], activation of p44/42 MAPK[173] and p70S6K, as well as GH receptor internalization and degradation and cleavage of the membrane GH receptor to release the extracellular domain of the GH receptor (GHBP)[204]. It has also been reported that PKC-d and PKC-e isoforms couple to the p44/42 MAP kinase pathway in 3T3-F442A cells and that the activation of p44/42 MAP kinases by GH specifically require PKC-d[173].

GH has also been reported to increase the intracellular free calcium concentration in a variety of cell types[202,205 208]. An increase in intracellular $[Ca^{2+}]$ can be achieved by two major mechanisms; either by influx of Ca^{2+} through voltage-dependent Ca^{2+} channels located in the plasma membrane or by mobilization of Ca^{2+} from intracellular stores[209]. GH-stimulated $[Ca^{2+}]$ increases apparently involves both Ca^{2+} influx and Ca^{2+} mobilization[210]. It is suggested that GH activates L-type calcium channels since the increase in intracellular $[Ca^{2+}]$ dependent on extracellular Ca^{2+} is blocked by verapamil and nimodipine[76]. The GH activated increase in intracellular $[Ca^{2+}]$ is apparently PKC dependent since the increase is blocked by the PKC inhibitor calphostin C and is also mimicked by the addition of DAG[202]. Verapamil has been demonstrated to prevent GH stimulated STAT5 mediated transcription[76], arachidonic acid production[176], expression of P4502C12 mRNA[176] and GH induced re-organization of the actin cytoskeleton[98]. Although the exact mechanism for the above mentioned phenomena remain to be elucidated, one of the most likely mechanism is through the intracellular calcium receptor calmodulin. It is interesting to note that calmodulin can directly bind to and activate Ral in a Ca^{2+}-dependent manner[211]. Our preliminary results also suggest that GH stimulated RalA activation is calcium dependent as GH stimulation of Ral activity is also blocked by verapamil (Zhu and Lobie, unpublsihed). The GH stimulated increase in intracellular $[Ca^{2+}]$ seems to require the C-terminal domain of the GH receptor[76] and it has been reported not to require the Box1 region required for the association of JAK2[76]. The related PRL receptor has been reported to require the Box 1 region for ligand stimulated increases in intracellular $[Ca^{2+}]$[212]. It is interesting to note that the initial release of intracellular Ca^{2+} in angiotensin II type 1 (AT1) receptor signaling results in the activation of Pyk2 and subsequent transactivation of the epidermal growth factor receptor (EGF-R)[213]. Whether a similar mechanism exists for GH induced tyrosine phosphorylation of EGF receptor remains to be investigated. Much work is therefore warranted to further characterize the downstream cellular targets of the GH stimulated elevation of intracellular $[Ca^{2+}]$.

III.7. STATs

As the most studied substrates of JAKs, signal transducer and activator of transcription (STAT) proteins are the only known family of transcription factors whose activity is regulated by tyrosine phosphorylation[214]. STAT proteins are recruited to the proximity of the activated JAKs by binding, through their SH2 domains, to phosphotyrosine-containing motifs on the cytoplasmic part of the receptor. Activated upon tyrosine phosphorylation by JAKs and followed by serine or threonine phosphorylation, the STAT molecules dissociate from the receptor with either homo-or hetero-dimerization through a reciprocal SH2 domain-phosphotyrosine interaction, translocate to the

nucleus, bind to their appropriate DNA response elements and stimulate transcription. The binding of STAT dimers to two adjacent DNA sites is co-operative and involves the formation of a tetrameric STAT-DNA binding complex. Many excellent detailed reviews exist on STAT signal transduction and we refer the reader to such reviews [8,30,208,214 215]. Suffice to say, GH has been demonstrated to utilize STATs 1, 3 [177,178,216], 5a and 5b[217 222] for the regulation of a variety of genes. GH has been demonstrated to stimulate the formation of STAT1 homodimers, STAT3 homodimers and STAT1/STAT3 heterodimers all binding to the SIE of the c-fos gene[177,216]. STAT5 is predominant STAT utilized by GH. The two forms of STAT5 (STAT5a and STAT5b) are encoded by two different genes, which exhibit ~90% identity in coding sequence and possess both overlapping and distinct functions in GH signal transduction[214 215]. Phenotypic analysis of STAT5a deficient mice revealed no effect on body growth, serum IGF-1 (the presumed mediator of the somatic effects of GH) levels and no alteration in the expression of GH regulated genes[223]. In contrast, generation of STAT5b deficient mice resulted in pronounced impairment of somatic growth in both sexes but especially in males [208,223] accompanied by a "feminization" of hepatic expression of testosterone 16a-hydroxylase CYP2D9 (normally high in males) and testosterone 15a-hydroxylase CYP2A4 (normally low in males)[208,223,224]. Thus STAT5b has been demonstrated to be involved in the sexually dimorphic responses of hepatic gene expression to male (pulsatile) and female (continuous) modes of GH secretion[219] and subsequent body growth. STAT5 is also utilized by GH to regulate insulin gene transcription[225] and STAT5b is required for GH stimulated lipolysis in adipose tissue[114,226].

GH activation of STATs 1, 3 and 5 requires the presence and activity of JAK2[167,227 229] although the mechanism differs for STATs 1 and 3 and STAT5[42,67,68,177 179]. STATs 1 and 3 are activated by the GH activation of JAK2 and do not require tyrosine residues in the carboxyl terminal intracellular domain of the GH receptor as does STAT5[230]. This is consistent with the fact that the GH receptor does not possess either the STAT1 or STAT3 association motifs such as exist in the IFN-g receptor or gp130 proteins[231]. The activation of JAK2 by GH may obviate such a requirement for receptor binding of STATs 1 and 3 since JAK2 itself has a STAT1 like association motif and 2 STAT3 association motifs. There is some controversy as to whether the membrane proximal tyrosine residues (333 and 338 in the rat GH receptor) are required for the STAT signaling response[42,232,233]. Apart from JAK2, STAT5 also utilizes tyrosine residues in the intracellular domain of the GH receptor for its full activation[230]. Although JAKs are the predominant kinases for STATs, receptor tyrosine kinases can also phosphorylate STATs in vivo without a requirement for JAK[234 237]. Whether these receptor tyrosine kinases and other non-receptor tyrosine kinases can phosphorylate the STATs in GH signaling remains to be identified.

Activation of STAT molecules has also been demonstrated to require serine phosphorylation in addition to tyrosine phosphorylation[238] with such serine phosphorylation provided by MAP kinase [238] or PI-3 kinase[143]. At least STAT3 has been demonstrated to associate directly with PI-3 kinase upon IFN-a stimulation[143] and GH stimulation (Zhu and Lobie, unpublished observation). Inhibition of MEK has also been demonstrated to prevent GH stimulated transcription mediated by STAT5a[185] but not nuclear translocation of STAT5a itself. It has also been suggested that STAT3 phosphorylated by

GH stimulation behaves as if it is also serine phosphorylated on sodium dodecyl sulphate polyacrylamide gel electrophoresis[231].

The GH activation of STAT molecules may be cell type specific despite the presence of a functional GH receptor. For example, GH was not observed to stimulate STAT1 or STAT3 activity in IM-9 lymphocytes despite the ability of IFN-g to activate these same STATs in the same cell line[239,240]. Some of this specificity may be provided by the interaction of different transcription factors binding to multiple response elements of the particular gene. One such example is the inhibition of transcription by STAT5 binding to the IRF-1 promoter[241]. Another is the regulation of STAT5 mediated transcription by a direct association with the glucocorticoid receptor[242].

The physiological roles of STATs in GH signal transduction have not been fully elucidated, although STAT-dependent pathways are generally believed to be utilized in cellular events such as cell proliferation, differentiation, and apoptosis[214]. STATs play a pivotal role in proliferation and apoptosis stimulated by other cytokines (such as EPO and IL-2) although this has not been reported for GH. Rather JAK kinase mediated activation of the p44/42 MAP kinase and p38 MAP kinase pathways have been demonstrated to be required for the propagation of GH initiated mitogenic signals[108]. Furthermore, JAK-2-STAT5 has been reported to be essential for GH-dependent differentiation of 3T3-F442A preadipocytes[243] and not the GH dependent activation of p44/42 MAPK and p70 S6 kinase. Thus the activation of JAKs and STATs by GH appear to be two independent but related events, which dictate two separate biological outcomes, the combination of which results in proliferation, survival and differentiation of cells.

Ⅲ.8. Negative Regulation of GH Signal Transduction

The precise control of cytokine receptor signal transduction requires the limitation of the magnitude and duration of the signal through negative regulation. The internalization/degradation pathway for the GHR-JAK2 complex[244,245] resulting in removal of the GH receptor from the cell surface and intracellular deactivation of signal transducing molecules appear to be critical for negative regulation of GH signal transduction.

The initial termination of GH signal transduction is presumably mediated by removal of cell surface GH receptor by internalization. The GH receptor is internalized by both the clathrin coated pit pathway [246] and caveolae[247]. It has been demonstrated that ubiquitination of the receptor is required for internalization and degradation[248] and a specific motif in the intracellular domain of the GH receptor is required for both ubiquitination and internalization of the receptor has been delineated[249]. The role of ubiquitination of the GH receptor in GH signal transduction is not apparent but may link the receptor to proteasomal degradation[250]. An intact ubiquitin system is required for GH activation of JAK-STAT[251] but GH stimulated STAT mediated transcription is normally activated by a ubiquitination deficient receptor mutant[252]. GH activation of PKC may act as a stimulus to remove receptor from the cell surface[253,254] as ligands that activate PKC also remove GH receptor from the cell surface with subsequent degradation[204]. It is interesting that PKC has also been demonstrated to interact with the structural component of caveolae, caveolin, which has been implicated in GH signal transduction [247,255]. The interaction of the GH receptor with caveolin may allow for the rapid desensitization and resensitization of the cellular response to GH by

allowing rapid removal and replacement of the receptor at the cell surface[247]. In any case, removal of the GH receptor from the cell surface diminishes the ability of GH to further alter cellular function.

There exist at least three families of proteins that inhibit JAK/STAT pathway signalling; phosphatases, suppressors of cytokine signaling (SOCSs) and protein inhibitors of activated STATs (PIAS).

Pre-incubation of cells with phosphatase inhibitors such as pervanadate[69,244] results in prolongation of GH stimulated JAK2 and STAT5 tyrosine phosphorylation indicative of phosphatase involvement in the de-activation of GH signal transduction. The Src homology 2 domain-containing protein-tyrosine phosphatase-1 (SHP-1) has been demonstrated to be activated by GH[51] and associates with phosphorylated JAK2[52]. GH also induces nuclear translocation and binding of SHP-1 to tyrosine-phosphorylated STAT5b, suggesting that this GH-activated phosphatase may be required for dephosphorylation leading to deactivation of nuclear STAT5b[51]. Interestingly, SHP-1 does not associate with the GH receptor[54,58] as might be expected from the EPO receptor signaling data but rather associates directly with JAK2[51]. In any case, SHP-1 deficient mice (motheaten mice) display prolonged GH stimulated tyrosine phosphorylation of JAK2 and STAT proteins compared to normal littermates[52]. However, a region of the GH receptor between amino acids 520 and 540 in the cytoplasmic domain has been identified to be required for attenuation of GH stimulated JAK-STAT signalling[52]. SHP-2 has been demonstrated to bind to tyrosine residues 487 and 595 (although tyrosine 595 appears to be the major site of interaction) of the GH receptor[256] and is responsible for tyrosine dephosphorylation of at least the GH receptor, JAK2 and STAT5b[256]. It should be noted that SHP-2 has been demonstrated to possess a dual role in GH signal transduction by its enhanced association with tyrosine phosphorylated SIRPa (signal-regulatory proteins) to increase transcription via the c-fos promoter[54]. It has also been postulated that proteasome mediated degradation is also involved in negative regulation of JAK2-STAT5 as the proteasome inhibitor MG132 sustained JAK2-STAT5 signalling[244].

The dual-specificity MAP kinase phosphatases (MKP) family and the serine/threonine protein phosphatase type 2 (PP2) family protein have been shown to be involved in the downregulation of MAP kinases, such as p44/42 MAP kinase, JNK/SAPK and p38 MAP kinase[257]. Their roles in GH signal transduction and interaction with STAT mediated transcription remain to be characterized.

More recently, two classes of proteins involved in the negative regulation of cytokine signaling have been demonstrated[214,258]. The first family of inhibitory proteins are suppressors of cytokine signaling (SOCSs) or cytokine-inducible SH2 containing proteins (CISs) of which eight members have been described[214,259]. Members of the family of SOCS proteins contain a central SH2 domain and a highly conserved C-terminal homology domain termed the SOCS box or the CH domain. SOCSs expression is induced by cytokine activation of the JAK/STAT pathway and they act as a negative feedback loop by subsequently inhibiting the JAK/STAT pathway either by direct interaction with activated JAKs or with cytokine receptors. These interactions are mediated by the SH2 domain and the SOCS box of the SOCS proteins[214,259]. SOCS proteins may serve as adaptor molecules to direct activated cell signaling proteins to the protein degradation pathway[260]. GH has been reported to stimulate mRNA expression of SOCS-1, SOCS-2, SOCS-3 and CIS[261 263]. Production of SOCS

proteins by GH is mediated by the activated STAT as a consequence of GH stimulation and therefore the negative regulation occurs after at least 30 minutes. The COOH-terminal SOCS box of the SOCSs is responsible for targeting these proteins for proteasomal degradation[214,263]. In an elegant study Ram and Waxman[263] have demonstrated that SOCS/CIS proteins can inhibit GH signal transduction to STAT5b by three distinct mechanisms distinguished by their molecular targets within the GHR-JAK2 signaling complex including ; (a) direct JAK2 interaction as exemplified by SOCS-1 ; (b) inhibition of JAK2 signalling via membrane proximal GH receptor tyrosine residues (SOCS-3) and (c) inhibition by utilization of membrane-distal tyrosine residues of the receptor (CIS and SOCS-2).

Protein inhibitor of activated STATs (PIAS) is another class of STAT inhibitors with differential preference for the individual STATs[214,264]. The interaction of PIAS proteins and STATs is cytokine dependent. Unlike the SOCS proteins, but similar to SHP-1, PIAS proteins are constitutively expressed and serve as a buffer to titrate the amount of activated STAT available in a particular cellular environment. Although PIAS-3 has been reported to be involved in the negative regulation of prolactin signal transduction[264,265], it remains to be determined if GH utilizes PIAS for the regulation of STAT mediated transcription.

VI. CONCLUSION

Rapid progress has been made recently in the definition of GH receptor signal transduction pathways. It is now apparent that many cytokines, including GH, share identical or similar signaling components to exert their cellular effects. Although many of these cellular effects are pleiotropic and/or universal to the cytokine receptor superfamily, some level of specificity does exist. The mechanisms by which the specific cellular effects of GH are achieved remain to be elucidated.

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