Identification of a JAK2-independent Pathway Regulating Growth Hormone (GH)-stimulated p44/42 Mitogen-activated Protein Kinase Activity

GH ACTIVATION OF Ral AND PHOSPHOLIPASE D IS Src-DEPENDENT*

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Tao Zhu‡, Ling Ling‡, and Peter E. Lobie§§

From the ‡Institute of Molecular and Cell Biology, 30 Medical Drive, Singapore 117609 and the §Department of Medicine, National University of Singapore, Singapore 119074, Republic of Singapore

We have demonstrated here that growth hormone (GH) stimulates the formation of the active GTP-bound form of both RalA and RalB in NIH-3T3 cells. Full activation of RalA and RalB by GH required the combined activity of c-Src and JAK2, both kinases activated by GH independent of the other. Activation of RalA and RalB by growth hormone did not require the activity of JAK2 per se. Ras was also activated by GH and was required for the GH-stimulated formation of GTP-bound RalA and RalB. Activation of RalA by GH subsequently resulted in increased phospholipase D activity and the formation of its metabolite, phosphatidic acid. GH-stimulated RalA-phospholipase D-dependent formation of phosphatidic acid was required for activation of p44/42 MAPK and subsequent Elk-1-mediated transcription. Thus, we report the identification of a JAK2-independent pathway regulating GH-stimulated p44/42 MAPK activity.

Due to lack of intrinsic kinase activity, members of the cytokine receptor superfamily, including the growth hormone (GH) receptor, recruit and activate non-receptor tyrosine kinases of the JAK family to relay their cellular signals (1). JAK2 has been reported to be the predominant JAK required for the initiation of GH signal transduction upon ligand binding to the GH receptor (2–4). To date, all identified downstream signaling pathways utilized by GH apparently require JAK2 activity (2–4). The only reported JAK2-independent effect of GH is entry via L-type calcium channels (5), although this has been disputed (6, 7). However, it is likely that other, as yet uncharacterized, signal transduction pathways stimulated by GH are activated independent of JAK2 activity.

The major groups of signaling molecules thus far identified to be activated by GH include the following: 1) other receptor (EGF receptor) (8) and non-receptor (c-Src, c-Fyn (9), and FAK (10)) kinases, although as in the case of the EGF receptor it may be used simply as an adapter protein; 2) members of the MAP kinase family including p44/42 MAP kinase (11, 12), p90 MAP kinase (13), and c-Jun N-terminal kinase/stress-activated protein kinase (9) and the respective downstream pathways; 3) members of the insulin receptor substrate (IRS) group including IRS-1, -2, and -3 which may act as docking proteins for further activation of signaling molecules including phosphatidylinositol 3-kinase (14); 4) small Ras-like GTPases (15); and 5) STAT family members including STATs 1, 3, 5a, and 5b (16, 17), which constitute one major mechanism for transcriptional regulation by GH.

Ras is a member of the Ras-like GTPase family (18, 19). This 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. The activation and inactivation of the Ras-like GTPases are controlled by conformational change because of a GTP-GDP binding cycle that is controlled by the following three different regulatory proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins, and guanine nucleotide dissociation inhibitors. In its GTP-bound state, Ras in turn interacts with distinct downstream effectors and initiates multiple signaling pathways, which include at least three downstream signaling cascades mediated by the Raf protein kinase (i.e. A-Raf, B-Raf, and Raf-1), Rap1GEF (i.e. RapGDS, Rif, and Rgl), and phosphatidylinositol (PI) 3-kinases (18–20).

Recent reports (20, 21) suggest that two other members of the Ras-like small GTPase family, namely RalA and RalB, possess pivotal roles in the control of cell proliferation, migration, differentiation, cytoskeletal organization, vesicular transport, and receptor endocytosis. Ral is also the member of the Ras-like GTPase family, and its activity is regulated by cycling between active GTP-bound and inactive GDP-bound states controlled through the direct binding of active Ras to Ral-specific GEFs. However, additional Ras-independent mechanisms also exist to stimulate the formation of GTP-bound Ral. For example (22, 23), RalA can be activated independently of Ras activation via its direct binding to Ca2+ alone (24) or to Ca2+-bound calmodulin in response to the elevated level of intracellular calcium (23). Moreover, PI 3-kinase (25) and Src-like kinases (25, 26) have also been implicated in Ral activation. Once activated, Ral further interacts with several other proteins that may function as its downstream effectors. RalA has been demonstrated to associate directly with phospholipase D1 (PLD1) via its N-terminal sequence and operates synergistically with another PLD1-interacting small GTPase, PLD2.
Arf, to activate PLD1 activity (27). Phospholipase D (PLD, including PLD1 and PLD2) is a widely expressed phospholipid-specific phosphodiesterase that hydrolyzes phosphatidylcholine, a major phospholipid in the cell membrane, to form phosphatidic acid (PA) and choline. PA can be further converted to diacylglycerol (DAG) and lyso-PA, both of which are the well-known intracellular mediators and extracellular messengers of multiple biological activities (28, 29). Two other proteins have also been reported that are known to interact with the GTP-bound form of RaLα, leading to RaLα-dependent cellular effects. The first is Ral-binding protein 1 or RaLB1 (also called RlIP76) (30), which is involved in receptor-mediated endocytosis (30, 31). RaLB1 is also a GTPase-activating protein for Cdc42, a Rho family member involved in actin cytoskeleton organization and filopodia formation in fibroblasts (30). The second is filamin, which serves as a downstream intermediate in Cdc42-mediated filopod production by its association with RaLa (32).

We have demonstrated here that GH stimulates the formation of the active GTP-bound form of both RaLα and RaLB in NIH-3T3 cells. Activation of RaLα and RaLB by growth hormone did not require the activity of JAK2 per se. However, full activation of RaLα and RaLB by GH required the combined activity of both c-Src and JAK2, both kinases activated by GH independent of the other. Activation of RaLα by GH subsequently resulted in the activation of PLD and the formation of phosphatidic acid (PA) and choline. PA can be further converted to specific phosphodiesterase that hydrolyzes phosphatidylcholine. This concentration of GH is within the physiological range for circulating rodent GH.

**3.2 JAK2 Immunoprecipitation—**Cells were lysed at 4 °C in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 0.5% Nonidet P-40, 0.1% phenylmethylsulfonyl fluoride, 1 μM leupeptin, 10 μg/ml aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride) for 30 min with regular vortices. Cell lysates were centrifuged at 14,000 × g for 15 min, and the resulting supernatants were collected, and protein concentration was determined. 800 μg of protein was used for each immunoprecipitation. Immunoprecipitations were performed by incubating lysates with 20 μl of gel slurry of anti-JAK2 protein A-agarose. The reaction mixture was gently rocked at 4 °C for 4 h. Immunoprecipitates were washed 3 times with ice-cold lysis buffer. The pellet was resuspended in 1× SDS sample buffer containing 50 mM Tris, pH 6.8, 2% SDS, 2% β-mercaptoethanol, and bromphenol blue. The samples were boiled for 10 min, and 25 μl aliquots were used for 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes using standard electroblotting procedures.

**Immunoblotting—**After preincubation with inhibitors for the indicated times and/or incubation with the indicated concentration of hGH for the appropriate duration, the cells were washed once with ice-cold PBS and lysed at 4 °C in an appropriate amount of lysis buffer. Cell lysates were dissolved and denatured in 1× SDS-PAGE sample buffer, and separation was achieved on 8–12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline with 0.1% Tween 20 for 1 h at 22 °C. The next day, the membranes were incubated with the appropriate antibody in PBS containing 1% non-fat dry milk at 4 °C overnight. After three washes with PBS, immunoblotting was determined by ECL according to the manufacturer's instructions. For reblotting, membranes were stripped by incubation for 30 min at 50 °C in a solution containing 62.5 mM Tris–HCl, pH 6.7, 2% SDS, and 0.7% mercaptoethanol. Blots were then washed for 30 min with several changes of PBS at room temperature. Efficacy of stripping was determined by re-exposure of the membranes to ECL. Thereafter, blots were reblocked and immunolabeled as described above.

**Src Kinase Assay—**Src kinase assays were performed as described according to the manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). Immunoprecipitates (150 μg of protein per sample) were derived from cells stimulated with hGH and incubated with 1 μg of Src polyclonal antibody at 4 °C for 2–4 h in a final volume of 500 μl. Immunocomplexes were collected by incubation with 20 μl of protein A/G plus-agarose for 1 h. Immunoprecipitates were washed 3 times with ice-cold lysis buffer. 10 μl (150 μl final concentration) of the immunoprecipitated protein was transferred to a microfuge tube, and 0.1 ml ice-cold sample buffer was added to a microcentrifuge tube and incubated for 10 min at 30 °C with agitation. 20 μl of 40% trichloroacetic acid was then added to precipitate peptides, and a 25-μl aliquot was transferred onto the center of a numbered P81 paper square. The assay squares were washed 5 times for 5 min each with 0.75% phosphoric acid and once with acetone. The squares were transferred to a scintillation vial, and 5 ml of scintillation mixture was added, and the level of radioactivity was determined in a scintillation counter. The sample that contains no enzyme serves as the background control.
formed according to the manufacturer's instructions. In brief, cells were lysed at 4 °C in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerol phosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1% phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin). The lysates were centrifuged at 15,000 × g for 15 min at 4 °C. The supernatant containing 200 µg of protein per sample was incubated overnight at least 4 h with an immobilized phospho-specific p44/42 MAP kinase (Thr<sup>202</sup>/Tyr<sup>204</sup>) monoclonal antibody (1:300 dilution) in a final volume of 500 µl in 1× lysis buffer. The pellets were washed twice with 500 µl of lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and twice with 500 µl of kinase buffer (25 mM Tris, pH 7.5, 5 mM glycerol phosphate, 2 mM dithiothreitol, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM MgCl<sub>2</sub>). The kinase reactions were performed in the presence of 2 µg of Elk-1 fusion protein and 200 µM ATP at 30 °C for 30 min. Elk-1 phosphorylation was selectively detected by Western immunoblotting using a chemiluminescence detection system and a specific phospho-Elk-1 (Ser<sup>389</sup>) antibody (1:1,000 dilution).

**Measurement of Phosphatidic Acid and PLD Activity**—Subconfluent cells were serum-starved and labeled overnight with [3H]palmitic acid (5 µCi/ml) in Dulbecco's modified Eagle's medium. After cells were stimulated with 50 nM hGH for the indicated time, the samples were placed on ice, rinsed with cold PBS, and the lipids extracted by the method of Bligh and Dyer (35). The dried samples were resuspended in chloroform/methanol/acetic acid (90:10:10, v/v/v) as the solvent with the unlabeled PA and phosphatidylethanol as lipid standards (Avanti Polar Lipids). For PLD activity assays, cells were stimulated with hGH in the presence of 1% ethanol to determine the total fatty acid label incorporated into lipid. PLD activity assays were also performed by use of the amplex red phospholipase kit from Molecular Probes (Eugene, OR). In brief, cells were lysed in the 1× reaction buffer with 1% Triton X-100 by several quick freeze-thaw cycles at −80 °C (10–15 min each). 100 µl of all −80 °C diluted samples containing 20 µg of total whole lysate was used to perform the assay. The fluorescence in a fluorescent microplate reader was measured using excitation detection at 540 nm and emission detection at 595 nm. Each point was triplicated, and the reading was corrected by subtracting the values derived from the non-PLD controls.

**Transient Transfection and Elk-1 Reporter Assay**—NIH-3T3 cells were cultured to 60–80% confluence for transfection experiments in 6-well plates (16). 0.2 µg of pCMVβ and 0.2 µg of reporter plasmid pFR-Luc were transfected together with 4 ng of the respective fusion trans-activator plasmid (pFA-Elk-1 or pPC2-dbd). For each well, 10–20 µl of Effectene for each µg of DNA was used as per the manufacturer's instructions. DNA-lipid complex was diluted to a final volume of 2 ml (for triplicate samples) with 2% fetal bovine serum medium and cells allowed to grow for 12–16 h. 50 nm hGH was added for an additional 24 h. The cells were washed in PBS and lysed with 200 µl of 1× lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100) by a freeze-thaw cycle, and lysate was collected by centrifugation at 14,000 rpm for 15 min. The supernatant was used for the assay of luciferase and β-galactosidase activity. The luciferase activities were normalized on the basis of protein content as well as on the β-galactosidase activity of pCMVβ vector. The β-galactosidase assay was performed with 20 µl of precleared cell lysate according to a standard protocol (13).

**Statistical Analysis and Presentation of Data**—All experiments were performed at least 3 times. Numerical data are expressed as mean ± S.D. Data were analyzed using the two-tailed t test or analysis of variance.

**RESULTS**

**hGH Stimulation of NIH-3T3 Cells Increases the Level of GTP-bound RalA and RalB**—We employed the GST-linked probe RLIP76-RBD (33), which recognizes only the active GTP-bound form of RalA and RalB and not the inactive GDP-bound form of these molecules, to determine the level of RalA-GTP and RalB-GTP in lysates of NIH-3T3 cells stimulated by hGH. hGH stimulation of NIH-3T3 cells resulted in the rapid formation of GTP-bound RalA and RalB which could be observed within 30 s of cellular stimulation with hGH (Fig. 1, A and C).

The hGH-stimulated formation of both RalA-GTP and RalB-GTP was biphasic, with the first peak of activity observed at 1–2 min after stimulation with hGH, followed by a decline to 15 min, and a second peak of GTP-bound RalA and RalB observed at 30 min, again followed by a decline to 60 min. hGH stimulation of NIH-3T3 cells did not alter RalA or RalB protein levels over the examined period of stimulation (Fig. 1, B and D). The hGH-stimulated formation of both RalA-GTP and RalB-GTP was also dose-dependent with stimulation of the GTP-bound forms of RalA and RalB first observed at 0.005 nM hGH and maximal stimulation from 5 to 50 nM hGH (Fig. 1, E and G). Thus RalA and RalB are two signaling molecules utilized by hGH to exert its effect on cellular function.

**hGH-stimulated Activations of JAK2 and c-Src Are Independent**—We next wished to determine the upstream kinases responsible for the hGH-stimulated conversion of RalA and RalB to the GTP-bound form. It was necessary, however, to first examine the potential interdependence between two GH activated kinases, namely JAK2 and c-Src (9, 36), after cellular stimulation with hGH. Neither the generic Src family kinase inhibitor PP1, nor the more specific Src kinase inhibitor PP2, nor the structurally related non-inhibitory PP3 affected JAK2 tyrosine phosphorylation stimulated by hGH (Fig. 2A). Simi-
Fig. 2. HGH-stimulated activation of JAK2 and c-Src are independent and parallel. A–E, hGH-stimulated activation of JAK2 is independent of c-Src activity. NIH-3T3 cells were stimulated with hGH after pretreatment and in the continued presence of vehicle, PP1, PP2, or PP3 as indicated, and cell extracts were prepared and processed for determination of tyrosine-phosphorylated JAK2 (A). Total JAK2 present in the JAK2 immunoprecipitates is indicated (B). NIH-3T3 cells were transiently transfected with the empty vector or an expression vector containing either wild type c-Src or kinase-dead c-Src (c-Src-KD) and stimulated with hGH, and cell extracts were prepared and processed for determination of tyrosine-phosphorylated JAK2 (C). Total JAK2 present in the JAK2 immunoprecipitates is indicated (D). The efficacy of wild type c-Src and kinase-dead c-Src overexpression is indicated by Western blot (E). F and G, hGH-stimulated activation of c-Src is independent of JAK2 activity. NIH-3T3 cells were stimulated with hGH after pretreatment and in the continued presence of vehicle, PP1, PP2, PP3, or AG490 as indicated, and cell extracts were prepared and processed for determination of Src kinase activity (F). NIH-3T3 cells were transiently transfected with the empty vector or an expression vector containing either kinase dead c-Src (c-Src-KD) or kinase-dead JAK2 (K882E) and stimulated with hGH, and cell extracts were prepared and processed for determination of Src kinase activity (G). H–K, AG490 and dominant negative JAK2 inhibit hGH-stimulated JAK2 tyrosine phosphorylation. NIH-3T3 cells were stimulated with hGH after pretreatment and in the continued presence of vehicle or AG490 as indicated, and cell extracts were prepared and processed for determination of tyrosine-phosphorylated JAK2 (H). Total JAK2 present in the JAK2 immunoprecipitates is indicated (I). NIH-3T3 cells were transiently transfected with the empty vector or an expression vector for dominant negative JAK2 (K882E) and stimulated with hGH, and cell extracts were prepared and processed for determination of tyrosine-phosphorylated JAK2 (J). Total JAK2 present in the JAK2 immunoprecipitates is indicated (K). The results presented are representative of a minimum of three (usually five) independent experiments.

Full Activation of RalA and RalB by hGH Requires Both JAK2 and c-Src—We proceeded to determine which of the two independently activated kinases described above were responsible for hGH-stimulated conversion of RalA and RalB to the GTP-bound form. We therefore first examined the effect of the JAK2 kinase inhibitor AG490 on hGH-stimulated conversion of RalA and RalB to the GTP-bound form. AG490 treatment of NIH-3T3 cells decreased the basal level of both RalA-GTP and RalB-GTP without alteration in the total cellular level of either RalA or RalB (Fig. 3, A–D). Treatment of NIH-3T3 cells with AG490 slightly diminished, but did not prevent, RalA-GTP and RalB-GTP formation stimulated by hGH. In contrast, AG490
completely prevented hGH-stimulated tyrosine phosphorylation of the JAK2 substrate STAT5 (data not shown). Similarly, forced expression of a kinase-deficient JAK2 slightly diminished, but did not prevent, RaLA-GTP and RaLB-GTP formation stimulated by hGH. Thus JAK2 is not required for hGH-stimulated conversion of RaLA and RaLB to the GTP-bound form. We next examined the effect of the generic Src family kinase inhibitor PP1, the more specific Src kinase inhibitor PP2, and the structurally related non-inhibitory PP3 on RaLA-GTP and RaLB-GTP formation stimulated by hGH. Both PP1 and PP2 abrogated, but did not completely prevent, RaLA-GTP and RaLB-GTP formation stimulated by hGH (Fig. 3E). PP3 did not affect hGH-stimulated RaLA-GTP and RaLB-GTP formation. The effect of PP1 and PP2 on inhibition of RaLA-GTP and RaLB-GTP formation stimulated by hGH was more potent compared with the effect of AG490 on hGH-stimulated GTP-bound RaLA and RaLB (compare Fig. 3, E and G to A and C). Forced expression of kinase-inactive c-Src also diminished, and to a greater extent than kinase-inactive JAK2, RaLA-GTP, and RaLB-GTP formation stimulated by hGH (Fig. 3, I and K). Because removal of the kinase activities of either JAK2 or c-Src only partially inhibited the formation of GTP-bound RaLA and RaLB stimulated by hGH, we therefore examined whether combined inhibition of JAK2 and c-Src would completely prevent RaLA-GTP and RaLB-GTP formation stimulated by hGH. Transient transfection of both kinase-inactive JAK2 and kinase-inactive c-Src cDNAs completely prevented hGH-stimulated RaLA-GTP and RaLB-GTP formation (Fig. 3, I and K). Thus, full activation of RaLA and RaLB by hGH required the combined activities of JAK2 and c-Src kinases.

Ras Activity Is Required for hGH-stimulated RaLA-GTP and RaLB-GTP Formation—Activation of RaLA and RaLB has been demonstrated previously (23, 38) to require Ras-dependent activation of RasGEFs thereby defining Ras as a Ras effector...
molecule. We therefore first examined the ability of hGH to stimulate the formation of Ras-GTP in NIH-3T3 cells. hGH stimulation of NIH-3T3 cells resulted in the rapid appearance of the GTP-bound form of Ras, maximal at 2 min and followed by a decline in the level of Ras-GTP such that at 30 min after stimulation with hGH the level of Ras-GTP returned to basal activity (Fig. 4A).

We next proceeded to determine the kinase dependence of the hGH-stimulated conversion of Ras to the GTP-bound form. We first examined the effect of the JAK2 kinase inhibitor AG490 on hGH-stimulated conversion of Ras to the GTP-bound form. AG490 treatment of NIH-3T3 cells completely prevented hGH-stimulated activation of Ras without alteration in the total cellular level of Ras (Fig. 4C and Fig. 4D). The Src kinase inhibitors PP1 and PP2 used above were without effect on the ability of hGH to stimulate the formation of GTP-bound Ras (Fig. 4E). Concordantly forced expression of a kinase-deficient JAK2 prevented Ras-GTP formation stimulated by hGH, whereas kinase-inactive c-Src was without effect on hGH-stimulated Ras activation (Fig. 4G). Thus, Ras activation by GH is JAK2-dependent.

To determine whether Ras activity was required for hGH-stimulated formation of RalA-GTP and RalB-GTP, we examined the ability of hGH to stimulate RalA-GTP and RalB-GTP formation in the presence of forced expression of RasN17. RasN17 forms a nonproductive complex with RasGEFs (22, 23) and therefore inhibits endogenous Ras activity. Forced expression of RasN17 abrogated the ability of hGH to stimulate the formation of both GTP-bound RalA and RalB (Fig. 4, K and M). Thus hGH-stimulated formation of RalA-GTP and RalB-GTP is Ras-dependent.

**Fig. 4.** HGH-stimulated formation of GTP-bound RalA and RalB is Ras-dependent. A and B, human GH stimulates the formation of GTP-bound Ras in NIH-3T3 cells. NIH-3T3 cells were stimulated with 50 nM hGH, and the GST-linked probe (GST)-Raf1-RBD, which recognizes the active GTP-bound form of Ras, was used to separate Ras-GTP from the inactive GDP-bound form of Ras. GTP-bound Ras was visualized by Western blot analysis as indicated (A). Total cellular Ras was also determined in total cell lysate by Western blot analysis as protein loading control (B). C–F, AG490 but not PP1, PP2, and PP3 inhibits hGH-stimulated formation of GTP-bound Ras. NIH-3T3 cells were stimulated with hGH after pretreatment and in the continued presence of vehicle or AG490, PP1, PP2, PP3, and GTP-bound Ras were visualized by Western blot analysis as indicated. Total cellular Ras (D and F) was also determined in total cell lysate by Western blot analysis as protein loading control. G–J, kinase-dead JAK2 but not kinase-dead c-Src inhibits hGH-stimulated formation of GTP-bound Ras. NIH-3T3 cells were transiently transfected with the empty vector or an expression vector containing either kinase-dead c-Src (c-Src-KD) or kinase-dead JAK2 (K882E) and stimulated with hGH. GTP-bound Ras (H) were visualized by Western blot analysis as indicated. Total cellular Ras (H) was also determined in total cell lysates by Western blot analysis as protein loading control. K–O, dominant negative Ras (RasN17) inhibits hGH-stimulated formation of the GTP-bound form of these molecules. GTP-bound RalA (K) and RalB (M) were visualized by Western blot analysis as indicated. Total cellular RalA (L) and RalB (N) were also determined in total cell lysates by Western blot analysis as protein loading control. The results presented are representative of a minimum of three (usually five) independent experiments.
may be upstream of, and influence, p44/42 MAP kinase activity stimulated by GH. We therefore examined the effect of forced expression of either wild type RalA or a dominant negative form of RalA (RalA28N) on the ability of hGH to activate p44/42 MAP kinase. As reported previously (40), hGH stimulation of NIH-3T3 cells resulted in a rapid time-dependent increase in the activity of p44/42 MAP kinase. Thus maximal activation of p44/42 MAP kinase activity was observed between 5 and 15 min after stimulation with hGH followed by a decline in activity to 60 min (Fig. 5A). Forced expression of wild type RalA slightly increased the basal activity of p44/42 MAP kinase activity and resulted in a prolonged activation of p44/42 MAP kinase activity in response to hGH. Thus, little or no diminution in p44/42 MAP kinase activity was observed 30–60 min after stimulation with hGH in comparison to the vector-transfected control (Fig. 5A). Forced expression of RalA at the different time points was demonstrated by the appearance of RalA at a slightly higher molecular weight (due to the presence of a FLAG tag) than endogenous RalA on Western blot analysis for RalA on the same whole cell lysates used for estimation of p44/42 MAP kinase activity (Fig. 5B). Forced expression of RalA28N resulted in decreased basal p44/42 MAP kinase activity, significantly less activation of p44/42 MAP kinase activity after GH stimulation, and shorter duration of the hGH-stimulated p44/42 MAP kinase activity (Fig. 5C). Similarly, forced expression of RalA28N at the different time points was demonstrated by the appearance of RalA28N at a slightly higher molecular weight (due to the presence of a FLAG tag) than endogenous RalA on Western blot analysis for RalA on the same whole cell lysates used for estimation of p44/42 MAP kinase activity (Fig. 5D). Thus, RalA is required for full activation of p44/42 MAP kinase activity by hGH in NIH-3T3 cells.

GH has been reported previously (13, 41) to stimulate transcription via Elk-1 in a p44/42 MAP kinase-dependent manner (13, 42). Furthermore, it has been reported that sustained activation of p44/42 MAP kinase is required for activation of Elk-1-mediated transcription (43). Because RalA overexpression resulted in sustained activation of GH-stimulated p44/42 MAP kinase, we examined the effect of forced expression of RalA and a RalA dominant negative mutant (RalA28N) on the ability of hGH to stimulate Elk-1-mediated transcription in NIH-3T3 cells. Forced expression of RalA increased the basal level of Elk-1-mediated transcription and dramatically increased the ability of hGH to stimulate transcription via Elk-1 (Fig. 5E). Forced expression of RalA28N reduced the basal level of Elk-1-mediated transcription and completely prevented hGH-stimulated Elk-1-mediated transcription (Fig. 5E). Thus RalA is required for full p44/42 MAP kinase activation by hGH and subsequent Elk-1-mediated transcription.

hGH Activates Phospholipase D—One of the proteins proposed to mediate the effects of Ral on cellular function is phospholipase D (27, 38, 44). We therefore examined whether hGH stimulation of NIH-3T3 cells would also result in an increase of phospholipase D activity. For determination of the effect of hGH on PLD activity, cells were stimulated with hGH in the presence of 1% ethanol, and PLD activity measured by the standard transphosphatidylation assay (27, 38, 44). hGH stimulation of NIH-3T3 cells resulted in a rapid rise in PLD activity, maximal at 5 min, and followed by a decline in activity to 60 min (Fig. 6A). We also measured the effect of cellular stimulation with hGH on PLD activity by use of the commercially available amplex red phospholipase D kit. As observed in Fig. 6, A and B, hGH stimulated an increase in PLD activity similar to that observed by use of TLC to determine PLD activity. Thus cellular stimulation with hGH resulted in an increase in PLD activity.

PLD catalyzes the hydrolysis of phosphatidylcholine and phosphatidylethanolamine to form phosphatidic acid (28, 29). We consequently next examined the effect of hGH stimulation of NIH-3T3 cells on PA production. Cells were serum-deprived and concomitantly incubated with [3H]palmitic acid before stimulation with hGH. The migration of PA in thin layer chromatography was identified against lipid standards, and the appropriate spot was removed and radioactivity determined. As is observed in Fig. 6C, hGH stimulation of NIH-3T3 cells resulted in a rapid rise in the level of PA with peak levels of PA observed at 5 min after stimulation. A sustained increase in the level of PA was observed to at least 60 min after stimulation.
GH activates phospholipase D and stimulates phosphatidic acid production. A and B, hGH activates phospholipase D activity in NIH-3T3 cells. NIH-3T3 cells were stimulated with hGH for the indicated times and processed for determination of PLD activity by either the standard transphosphatidylation assay (A) or by use of the commercially available amplex red phospholipase D kit (B). C, hGH stimulates phosphatidic acid production. NIH-3T3 cells were serum-deprived and concomitantly incubated with [3H]palmitic acid before stimulation with hGH. The migration of PA in thin layer chromatography was identified against lipid standards, and the appropriate spot was removed and radioactivity determined. D, hGH activation of PLD is RaLA-dependent. NIH-3T3 cells were transiently transfected with the expression vectors for wild type RaLA or dominant negative RaLA (RaLA28N), stimulated with hGH, and processed for PLD activity as indicated under "Experimental Procedures." Data presented are mean ± S.E. of triplicate determinations. Experiments were repeated a minimum of 3 (usually 5) times.

with hGH. Thus hGH stimulates PA production in NIH-3T3 cells (Fig. 6C).

hGH Activation of PLD Is RaLA-dependent—To determine whether hGH-stimulated activation of phospholipase D is RaLA-dependent, we determined the effect of forced expression of either wild type RaLA or a dominant negative form of RaLA (RaLA28N) on the ability of hGH to activate PLD. As observed in Fig. 6D, transfection of wild type RaLA cDNA did not significantly alter the basal level of PLD activity but significantly enhanced the hGH-stimulated increase in PLD activity. Conversely, transfection of NIH-3T3 cells with a dominant negative form of RaLA, although not altering the basal level of PLD activity, abrogated the ability of hGH to stimulate increases in PLD activity (Fig. 6D). Thus hGH-stimulated activation of PLD is RaLA-dependent.

PLD Activity Is Required for hGH-stimulated p44/42 MAP Kinase Activity and Elk-1-mediated Transcription—PLD has been demonstrated to exist in a complex with RaLA and the small G protein ADP-ribosylation factor-1 (ARF1) (27). Inhibition of ARF1 with the fungal metabolite BFA has been demonstrated to prevent the activation of PLD by extracellular stimuli (28, 29, 46). We therefore first examined the effect of BFA on the ability of hGH to stimulate p44/42 MAP kinase activity. As observed in Fig. 7A, BFA in concentrations ranging from 1 to 50 μg/ml effectively inhibited the activation of p44/42 MAP kinase by hGH. To demonstrate that the inhibition of hGH-stimulated p44/42 MAP kinase by BFA was specifically due to inhibition of PLD-dependent PA production, we added exogenous PA at the same time as BFA and examined p44/42 MAP kinase activity in response to cellular stimulation with hGH. Exogenously added PA, in the presence of 50 μg of BFA which effectively prevented p44/42 MAP kinase activation by hGH, restored hGH-stimulated p44/42 MAP kinase activity (Fig. 7B). Thus BFA inhibition of hGH-stimulated p44/42 MAP kinase activity by hGH was specifically due to inhibition of PA production.

To verify the results obtained with BFA, we therefore examined the effect of forced expression of PLD1, an enzymatically inactive form of PLD1 (PLD1-K898R), PLD2, and an enzymatically inactive form of PLD2 (PLD2-K758R) on hGH-stimulated p44/42 MAP kinase activity. Forced expression of PLD1 increased the basal level of p44/42 MAP kinase activity and also increased hGH-stimulated p44/42 MAP kinase activity (Fig. 7C). The enzymatically inactive form of PLD1 unexpectedly also increased basal p44/42 MAP kinase activity but prevented any stimulation of p44/42 MAP kinase activity by hGH (Fig. 7C). Forced expression of PLD2 did not significantly alter the basal activity of p44/42 MAP kinase but significantly enhanced p44/42 MAP kinase activity stimulated by hGH (Fig. 7C). The enzymatically inactive form of PLD2 did not significantly alter basal p44/42 MAP kinase activity and prevented the hGH-stimulated increase in p44/42 MAP kinase activity (Fig. 7C). The forced expression of PLD1, PLD1-K898R, PLD2, and PLD2-K758R was verified by Western blot analysis (Fig. 7D).

We next examined the effect of forced expression of PLD1, an enzymatically inactive form of PLD1 (PLD1-K898R), PLD2, and an enzymatically inactive form of PLD2 (PLD2-K758R) on hGH-stimulated Elk-1-mediated transcription. Forced expression of PLD1 increased both basal and hGH-stimulated Elk-1-mediated transcription. Forced expression of PLD1 increased basal level of Elk-1-mediated transcription and prevented an hGH-stimulated increase in Elk-1-mediated transcription. Forced expression of PLD2 did not increase the basal level of Elk-1-mediated transcription but substantially enhanced hGH-stimulated Elk-1-mediated transcription when compared with the vector-transfected control. Forced expression of PLD2-K758R completely prevented hGH-stimulated Elk-1-mediated transcription (Fig. 7G).

A RaLA-PLD Pathway Is Required for hGH-stimulated Activation of Elk-1-mediated Transcription—We have demonstrated above that forced expression of RaLA dramatically enhanced the ability of hGH to stimulate Elk-1-mediated transcription and that activation of PLD in response to cellular stimulation with hGH was RaLA-dependent. It was therefore required to demonstrate that the RaLA enhancement of hGH-stimulated Elk-1-mediated transcription was PLD-dependent. We therefore tested whether the enzymatically inactive PLD2 (PLD2-K758R) could inhibit the increase in hGH-stimulated Elk-1-mediated transcription consequent to forced expression of RaLA. As observed above, transfection of RaLA cDNA dramatically enhanced both the basal and hGH-stimulated Elk-1-mediated transcription, and PLD2-K758R prevented any hGH-stimulated increase in Elk-1-mediated transcription. When both RaLA and PLD2-K758R were transfected together, an increase in the basal level of Elk-1-mediated transcription was evident as is observed for forced expression of RaLA alone, but no hGH-stimulated increase in Elk-1-mediated transcription was observed (Fig. 8). Thus, RaLA enhancement of hGH-stimula-
We have demonstrated here that hGH stimulation of NIH-3T3 results in a rapid and biphasic activation of the small Ras-like GTPases RalA and RalB. Similar biphasic activation of Ral (30) and other small Ras-like GTPases including Ras (25, 47) and Rap1 (48) has been reported in other systems. It has been postulated that this phenomenon is due to the differential input dynamics of upstream signals and/or differential feedback from downstream effector molecules. For example, it has been reported that biphasic activation of Ras by endothelin-1 was linked to the sequential activation of two downstream pathways, p44/42 MAP kinase and PI 3-kinase (47). The decrease in Ras activity following the first peak of Ras activity stimulated by endothelin-1 elicits a negative feedback through p44/42 MAP kinase-dependent Sos1 phosphorylation, whereas the second peak of Ras activation by endothelin-1 is facilitated by persistent tyrosine phosphorylation of SHC (47). The implication of such a bipartite activation of Ral and its potential contribution to GH signal transduction requires further investigation.

We have demonstrated here that hGH-stimulated activation of c-Src is independent of the activity of JAK2. This is the first reported example that a kinase utilized by GH does not require the activity of JAK2. Other kinases utilized by GH, such as focal adhesion kinase, have been reported to associate with and require the activity of JAK2 (10). We have reported previously (9) that GH also activates another Src kinase, c-Fyn, although the dependence of its activation on JAK2 was not investigated. It is also likely that other members of the Src family of kinases activated by GH, such as c-Fyn, are activated independent of JAK2-mediated transcription requires the activity of at least PLD2.

**DISCUSSION**

We have demonstrated here that hGH stimulation of NIH-3T3 results in a rapid and biphasic activation of the small Ras-like GTPases RalA and RalB. Similar biphasic activation of Ras (30) and other small Ras-like GTPases including Ras (25, 47) and Rap1 (48) has been reported in other systems. It has been postulated that this phenomenon is due to the differential input dynamics of upstream signals and/or differential feedback from downstream effector molecules. For example, it has been reported that biphasic activation of Ras by endothelin-1 was linked to the sequential activation of two downstream pathways, p44/42 MAP kinase and PI 3-kinase (47). The decrease in Ras activity following the first peak of Ras activity stimulated by endothelin-1 elicits a negative feedback through p44/42 MAP kinase-dependent Sos1 phosphorylation, whereas the second peak of Ras activation by endothelin-1 is facilitated by persistent tyrosine phosphorylation of SHC (47). The implication of such a bipartite activation of Ral and its potential contribution to GH signal transduction requires further investigation.

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JAK2. The related hormone prolactin, which also utilizes JAK2 for its signal transduction, has also been demonstrated previously (6) to stimulate c-Src-independent activation of JAK2. Other examples of JAK-independent activation of kinases by members of the cytokine receptor superfamily includes interleukin-3 activation of Sre (49) and erythropoietin activation of Lyn (50). Interestingly it has been reported (51) that angiotensin II stimulates an association between the N terminus of JAK2 and the SH2 domain of c-Src which is dependent on the activity of JAK2. Whether such an association was also required for activation of Sre by angiotensin II was not demonstrated (51). In any case such an association would allow JAK2 and c-Src to be spatially co-located and may facilitate synergetic interactions where common signaling molecules are involved. In this regard it is relevant to note that GH stimulates the association of JAK2 and FAK, and FAK is one component of a multiprotein signaling complex centered around CrkII and also including c-Src (9). FAK has also been reported to be a Sre substrate (10). In this case, however, at least the activity of JAK2 was not required for GH-stimulated activation of c-Src. We cannot exclude the possibility that the JAK2 molecule itself, and not JAK2 kinase activity, may be required for the activation of c-Src. However, prolactin is able to activate c-Src in the absence of the proline-rich Box1 region of the prolactin receptor required for the activation of JAK2, and therefore JAK2 association with the prolactin receptor is not required for prolactin-stimulated Sre activity (6). Thus, there may exist multiple independent parallel pathways by which GH could affect cellular function. It remains to be determined what the contribution of JAK2-independent signaling pathways will be to the final cellular effects of GH. Analysis of the genetic targets of the different signaling pathways by cDNA microarray may prove useful in this regard and is in progress.

We have demonstrated here that full activation of RalA and RalB by GH requires the combined activity of both c-Src and JAK2. It is interesting to note however that the impairment in Ral activation by inhibition of c-Src is considerably greater than that observed by inhibition of JAK2. This phenomenon was observed with utilization of both pharmacological inhibitors and cellular expression of the respective kinase-deficient molecules. It is therefore apparent that formation of GTP-bound Ral by GH is predominantly mediated by GH-stimulated c-Src activity. The requirement for JAK2 activity for full GH-stimulated activation of RalA and RalB is apparently due to the exclusive JAK2-dependent activation of Ras by GH (see below). Thus we have identified two signaling molecules (RalA and RalB) that can be activated by GH, albeit to a lesser extent, in the absence of JAK2 activity. Both fMet-Leu-Phe and platelet-activating factor activation of Ral in neutrophils have also been demonstrated to be partially dependent on c-Src (25). Furthermore, RalA has been demonstrated previously (44, 58) to mediate activation of PLD in v-Src-transformed cells. It is interesting to note that Ral has also been demonstrated to regulate the activity of c-Src in response to cellular stimulation by EGF (52). It is therefore possible that Ral participates in regulating the final “output” of the GH-stimulated multiprotein signaling complex centered around CrkII and containing c-Src (9) in addition to functioning in the linear pathway we have described here. Further support for a role of c-Src in GH signal transduction is the ability of Csk (Src-inactivating kinase) to inhibit GH-stimulated p44/42 MAP kinase activity (53), and this observation is likely to be mediated by the Src-Ral-PLD pathway we have described here (also see below for discussion).

Another small GTPase, Ras, has been demonstrated previously (15, 39) to be activated by GH, and we have also observed here that GH stimulates the rapid formation of GTP-bound Ras in NIH-3T3 cells. Ras proteins are activated by RalGEFs which are themselves activated by direct binding to Ras (18, 20). Transient transfection of the dominant negative Ras mutant RasN17 attenuated GH-stimulated formation of GTP-bound RalA and RalB suggestive that GH activation of RalA and RalB is also Ras-dependent. Ras has further been demonstrated to be activated by Ras-independent pathways (22–24), and the failure of RasN17 to inhibit completely GH-stimulated formation of RalA-GTP and RalB-GTP indicates that GH also utilizes Ras-independent pathways to participate in Ral activation. Ras has been reported to be activated by Rap1 (33). In addition, both Rap1 (54) and RalA (33, 34) can be activated in response to an elevated level of intracellular calcium. It has been reported that Src-like kinase activity is required for GH-stimulated calcium influx (7, 55). As GH-stimulated Rap activation is also Src kinase-dependent, it would be reasonable to propose that GH-stimulated Rap activity might also be mediated via Ca2+ influx. By use of calcium channel inhibitors, we have indeed demonstrated that GH activation of RalA and RalB is also dependent on Ca2+ influx via L-type calcium channels.2 Whether Rap1 is also involved in GH-stimulated Ral activation requires further investigation. We have observed that GH stimulation of Chinese hamster ovary cells stably transfected with GH receptor cDNA (CHO-GHR-(1–638)) results in a potant activation of Rap1, whereas minimal activation of Rap1 by GH is observed in NIH-3T3 cells.3 We have also observed a similar preferential activation of c-Jun N-terminal kinase by GH in CHO-GHR-(1–638) cells in comparison to NIH-3T3 cells due to a relative deficiency of CrkII (9, 40), and Rap1 activation has been demonstrated previously (56) to be CrkII-dependent. In any case, it remains to be determined if Rap1 will participate in the activation of RalA or RalB in NIH-3T3 cells. Other Ras-related molecules such as TC21 have also been demonstrated to activate Ral (57). Further work should delineate the signaling

2 T. Zhu, L. Ling, and P. E. Lobie, unpublished data.
3 L. Ling, T. Zhu, and P. E. Lobie, manuscript in preparation.
molecules downstream of JAK2 and c-Src utilized by GH to stimulate the formation of GTP-bound RaLa and RaIB.

It is interesting to note in this study that the overexpression of wild type RaLa resulted in an extended activation of p44/42 MAP kinase activity but did not increase the maximal level of GH-stimulated p44/42 MAP kinase activity. An analogous situation has been described for nerve growth factor-stimulated activation of p44/42 MAP kinase in PC12 cells (43). In that example, Ras was required for the initial activation of p44/42 MAP kinase by nerve growth factor, and the small GTPase Rap1 maintained the activation of p44/42 MAP kinase (43). Similarly, the Rap1-sustained activation of p44/42 MAP kinase by nerve growth factor was required for full activation of Elk-1-mediated transcription (43). We also observed that overexpression of RaA resulted in dramatically increased Elk-1-mediated transcription stimulated by GH indicative that RaA is a pivotal component in the mediation of the effects of p44/42 MAP kinase activation by GH. Analogously, the decreased GH-stimulated activation of p44/42 MAP kinase observed upon overexpression of the dominant negative RaA28N may simply be due to the inability of the cell to maintain p44/42 MAP kinase in an activated form rather than any deficit in activation. In any case, overexpression of dominant negative RaA28N resulted in the absence of GH-stimulated Elk-1-mediated transcription. Thus RaA regulation of p44/42 MAP kinase activity to produce sustained high level activation would be required for the full transcriptional program initiated upon activation of p44/42 MAP kinase by GH.

We have demonstrated here that GH stimulation of NIH-3T3 cells results in the activation of PLD and the subsequent generation of phosphatidic acid in the cells. RaA has been demonstrated previously (44) to mediate activation of PLD in v-Src-transformed cells. Thus, overexpression of RaA potentiated PLD activation by v-Src, and dominant negative RaA inhibited PLD activity in both v-Src- and v-Ras-transformed cells (55). We have analogously demonstrated that hGH stimulates the activation of RaA in both a c-Src- and Ras-dependent manner and that the hGH-stimulated activation of PLD is indeed RaA-dependent. The association of RaA and Arf has been demonstrated previously (27) to be required for increased PLD activity. PLD-catalyzed hydrolysis of phospholipids results in the generation of PA. The generation of PA by GH was demonstrated to be essential for GH-stimulated p44/42 MAP kinase activation as BFA (which prevents PLD activation and subsequent PA production by inhibiting Arf GTP-GDP exchange) dramatically diminished GH-stimulated p44/42 MAP kinase activation. Furthermore, the pretreatment of cells with PA significantly reversed the inhibition of GH-stimulated p44/42 MAP kinase activation by BFA. Transfection of dominant negative mutants of PLD (PLD1-K898R or PLD2-K758R) also prevented GH-stimulated p44/42 MAP kinase activation and Elk-1-mediated transcription. It is therefore apparent that PA serves as an effector generated as a result of PLD activation for p44/42 MAP kinase activation by GH. It has been proposed that PLD and its PA product mediate agonist-dependent Raf-1 translocation to the plasma membrane and the subsequent activation of the p44/42 MAP kinase pathway (46). The recruitment of Raf-1 to membranes is mediated by direct interaction of Raf-1 with PA and is independent of association with Ras (46, 59). It remains to be determined whether the requirement of PA for GH-stimulated p44/42 MAP kinase activation is due to a similar mechanism. It is possible that Raf and Raf-1 may independently activate the p44/42 MAP kinase pathway as both Raf and RaIGDS signaling independently stimulate hTBP promoter activity in a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase activation-dependent manner (60). Other GH-stimulated direct PA-dependent cellular events remain to be determined. PA has been proposed to be a potent activator of signaling molecules such as tyrosine kinases, GTPase-activating protein, PI 4-kinase (which produces the PLD cofactor phosphatidylinositol 4,5-bisphosphate), in addition to Raf (61). PA has further linked to Ca^{2+} signaling (62) and to superoxide anion production through NADPH oxidase (63).

Alternatively, once produced, PA may be hydrolyzed by PA phosphohydrolase to produce DAG with resultant activation of PKC (28, 29, 61). Although PKC has been demonstrated to be required for GH-stimulated activation of p44/42 MAP kinase in other cellular systems (3), we have observed no PKC dependence of GH-stimulated p44/42 MAP kinase activation here.4 Thus, Raf is unlikely to regulate GH-stimulated p44/42 MAP kinase activity by DAG generation from PA and subsequent PKC activation. PA can also be converted to lyso-PA and arachidonic acid (AA) by the action of phospholipase A2 (28, 29). GH has been demonstrated previously (64) to activate PLA2, and activation of PLA2 by GH increases the level of AA and subsequent formation of AA metabolites. Inhibition of PLA2 partially inhibits GH-stimulated p44/42 MAP kinase activation,2 suggestive that the catalytic action of PLA2 on PA is involved in the Raf-PLD-p44/42 MAP kinase pathway described here.

Raf has been implicated in the control of cell proliferation and Ras-mediated oncogenic transformation (19, 44, 57). For example, expression of RafGEFs or activated Raf proteins can cooperate with activation of other Ras effector cascades to result in cellular transformation (65). Although GH stimulation of NIH-3T3 cells results in a marked increase in p44/42 MAP kinase activity, there is little increase in cell number in response to exogenous GH. Thus activation of Ras and Raf per se will not necessarily result in mitogenesis nor oncogenic transformation. In other cellular systems, such as the mammary carcinoma cell, both autocrine and exogenously added hGH result in p44/42 MAP kinase-dependent mitogenesis (66, 67). In this regard it is interesting that autocrine hGH production by mammary carcinoma cells results in a dramatic increase in cyclin D1 transcription (68), and Raf has been demonstrated previously to regulate cyclin D1 gene transcription through NF-{kappa}B (65). Furthermore, overexpression in NIH-3T3 cells of a Ras effector mutant that activates RafGEF but not Raf or PI 3-kinase resulted in the formation of a metastatic and invasive phenotype (69). We have observed that autocrine production of hGH in mammary carcinoma cells also results in an invasive phenotype,5 and it is likely that Raf may be required for such an effect. PLD1 has also been demonstrated to contribute to cellular proliferation as transfected PLD1 results in the oncogenic transformation of 3Y1 cells overexpressing the EGF receptor (45).

In conclusion, we have demonstrated that GH stimulation of NIH-3T3 cells results in JAK2-independent formation of GTP-bound RaA and RaB with subsequent regulation of GH-stimulated p44/42 MAP kinase activity through PLD. A diagrammatic summary of this pathway is provided in Fig. 9. The identification of JAK2-independent activation of specific signaling pathways by GH will dramatically increase our understanding of the repertoire of signaling molecules utilized by GH to achieve its pleiotropic cellular effects.

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