RhoA/ROCK Activation by Growth Hormone Abrogates p300/Histone Deacetylase 6 Repression of Stat5-mediated Transcription*

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We demonstrate here that growth hormone (GH) stimulates the activation of RhoA and its substrate Rho kinase (ROCK) in NIH-3T3 cells. GH-stimulated formation of GTP-bound RhoA requires JAK2-dependent dissociation of RhoA from its negative regulator p190 RhoGAP. Inactivation of RhoA does not affect GH-stimulated ROCK activities are required for GH-stimulated, Stat5-mediated transcription. We also demonstrate that RhoA is the pivot for cAMP-dependent protein kinase inhibition of GH-stimulated, Stat5-mediated transcription. We have therefore provided a novel mechanism by which a Ras-like small GTPase, RhoA, can regulate Stat5-mediated transcription.

RhoA belongs to the Rho subfamily of the Ras-related, small GTPase superfamily that consists of five groups as follow: Ras, Rho, Rab, Arf, and Ran (1). Twenty mammalian Rho family members have been identified. Among them, RhoA, Rac, and Cdc42 have been extensively studied (1). RhoA has been demonstrated to be involved in diverse cellular processes including cytoskeleton organization, hormone secretion, gene transcription, cell cycle progression, and cell transformation (2). The activity of RhoA is regulated by three classes of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP dissociation inhibitors (GDIs) (1).

Numerous RhoA GEFs have been reported, including Lfc, Lse, Lb, Net1, Dbl, Vav, and a newly identified GEF family consisting of PDZ-RhoGEF, p115 RhoGEF, and LARG (3, 4). p190 RhoGAP is the best known GAP for RhoA, containing an NH2-terminal GTP binding domain and a C-terminal domain possessing the GAP activity (5). p190 RhoGAP is pivotal for RhoA inactivation in several signaling pathways (6–8). RhoGDIs negatively regulate RhoGTPases activity by their extraction from plasma membrane and subsequent formation of inactive cytosolic complexes with them (9).

RhoA activity can be modulated by tyrosine kinases or serine kinases. Tyrosine kinase Src negatively regulates RhoA by activation of p190 RhoGAP (7). Another kinase modulating RhoA activity is the serine kinase PKA that has been reported to inactivate RhoA through phosphorylation of RhoA on serine 188 (10). Upon activation, RhoA interacts with and stimulates effector proteins, including Rho kinase (ROCK), mDia, protein kinase N, and phosphatidylinositol 4-phosphate 5-kinase (11). As the most important effector, ROCK is implicated in the various cellular functions downstream of RhoA, such as actin cytoskeleton organization, transformation, and regulation of transcription (11, 12).

The first demonstrated transcriptional event regulated by RhoA was a fos promoter-transcription factor-mediated transcription of the c-fos promoter in NIH-3T3 cells (13). To date, several other RhoA-regulated transcription factors have been identified, including NF-κB, Stat3, Stat5, ATF2, Max, and CHOP (14). Stats are important regulators in cytokine signaling and are responsible for transcriptional activation of target genes that control proliferation, differentiation, and survival (15). RhoA triggers simultaneous phosphorylation at both tyrosine and serine residues of Stat3 and subsequent activation of Stat3 essential for oncogenic RhoA-mediated transformation (14). RhoA also promotes tyrosine phosphorylation and serine dephosphorylation of Stat5A with a concomitant increase in Stat5A activity to mediate morphological transition induced by oncogenic RhoA (16). Although the mechanism for RhoA to regulate gene expression remains unclear, recent studies suggest that it is possibly achieved through the modulation of transcriptional cofactor p300/CBP by RhoA. It has been reported (17) that RhoA inhibits inducible nitric-oxide synthase expression via negative regulation of the NF-κB-CBP/p300 pathway. The antagonistic relationship between RhoA and the transcriptional cofactor CBP/p300 suggested above is consistent with the previous findings (18) that RhoA inhibited whereas RhoGDI stimulated CBP/p300-mediated estrogen receptor-dependent transactivation.

Growth hormone (GH) regulates proliferation, differentiation, apoptosis, and chemotaxis in various cell types (19). The
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Predominant mechanism by which GH exerts its cellular function is through regulation of gene transcription (19). Stat5, existing as the two isoforms 5A and 5B encoded by different genes, is a major mediator of GH-dependent transcription (19). GH-stimulated activation of Stat5 requires phosphorylation of a single tyrosine residue in Stat5 by Jak2 (20). In addition to tyrosine phosphorylation, the transcriptional activity of Stat5 can be regulated by serine phosphorylation, either positively or negatively dependent on promoter context (21). Upon activation by GH, Stat5 binds to interferon-γ-activated sequence-like elements (GLE) in the promoter of several genes, such as the serine protease inhibitor Sp1, insulin I, cytochrome P450 3A, and β-casein genes (22). Recently, chromatin remodeling has also been proposed to regulate GH-stimulated Sp1 2.1 gene expression (23). Ras-like small GTPases have also been identified to be required for certain transcriptional events stimulated by GH (24–26). Ras and RalA are required for GH-stimulated p44/42 MAP kinase activity and subsequent Elk-1-mediated transcription (25, 26). Rap1 constrains the activity of GH-stimulated p44/42 MAP kinase and subsequent Elk-1-mediated transcription through inactivation of RalA (24). GH stimulation of Rap1 also serves as a switch to activate CrkII-mediated transcription through inactivation of RalA (24). GH-mediated transcription (25, 26). Rap1 constrains the activity of GLE elements (GLE) in the promoter of several genes, such as the negatively dependent on promoter context (21). Upon activation of the transcription complex.

Transcriptional activation is generally correlated with histone acetylation by histone acetyltransferase (HAT) complexes, and repression is correlated with deacetylation by HDAC complexes (29). The transcription cofactor p300 and its closely related protein CBP, which are ubiquitous and critical regulators of transcription, possess intrinsic HAT activity and can activate transcription through histone acetyltransferase-dependent chromatin remodeling or acetylation of transcription activators, general transcription factors, and chromatin-associated proteins (30). Alternatively, they serve as adaptors to bridge transcription factors with the basic transcription machinery to facilitate transcriptional initiation (30). However, p300 can also repress transcription. It has been reported that p300 down-regulates c-Myc with subsequent cell cycle arrest at G1/S transition (31). A recent report (32) has also identified a repression domain in p300 termed CRD1, which mediates repression of p53-dependent transcription by recruitment of HDAC6 to the transcription complex. Here we demonstrate that cellular stimulation with GH results in the activation of RhoA and its substrate ROCK in NIH-3T3 cells. The activation of RhoA by GH is achieved by Jak2-dependent dissociation of RhoA from p190 RhoGAP. We further demonstrate that GH utilizes the RhoA-ROCK pathway to stimulate Stat5-mediated transcription through RhoA-dependent prevention of recruitment of HDAC6 by p300. We also identify PKA as a negative regulator of GH-stimulated, Stat5-mediated transcription, and this cellular effect of PKA requires the PKA phosphorylatable serine residue 188 of RhoA.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human growth hormone (hGH) was a generous gift of Novo Nordisk (Singapore). The monoclonal antibodies against p190 RhoGAP and ROCK were obtained from Transduction Laboratories (Lexington, KY). The polyclonal antibodies against Jak2, ROCK, or Stat5a/b, the monoclonal antibody against RhoA, and protein A/G plus agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). GFP monoclonal antibody was purchased from Clontech (Palo Alto, CA). The monoclonal antibodies against Src, p300, phospho-Stat5a/b (Tyr-694/Tyr-699), Myc tag (clone 9E10), or phosphotyrosine (4G10) and the polyclonal antibody against phospho-MYPT (Thr-696) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-mouse IgG conjugated to fluorescein isothiocyanate and anti-rabbit IgG conjugated to horseradish peroxidase and the ECL kit were purchased from Amersham Biosciences. The p44/42 MAP kinase assay kit was purchased from New England Biolabs (Beverly, MA). The biotin DNA labeling kit was from Pierce. The transfection reagent Effectene™ was from Qiagen (Hilden, Germany). The complete protease inhibitor mixture tablets were purchased from Roche Diagnostics. The QuikChange® site-directed mutagenesis kit was purchased from Stratagene (La Jolla, CA). The β-galactosidase enzyme activity assay system was from Promega (Madison, WI). The [3H]acetetyl-CoA was from PerkinElmer Life Sciences. Myristoylated PKA inhibitor was from Calbiochem, trichostatin A was from Sigma, and forskolin and 8-Br-cAMP were from Merck. DAPI was from Molecular Probes (Eugene, OR). P81 nondegradable paper squares were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). All other chemicals were obtained from Sigma.

pGEX-3X-C21 construct containing the RBD of rhotekin, the pCAG-Myc-Rock construct, and the GFP-C3 exoenzyme expression vector were the generous gifts of Dr. Shah Narumiya (Kyoto, Japan). The wild type RhoA cDNA was purchased from Upstate Biotechnology, Inc. The kinase-defective mutant DNA constructs for c-Src and JAK2 were generously provided by Dr. Joan S. Brugge (Boston, MA) and Dr. Olli Silvennoinen (Tampere, Finland), respectively. The wild type p190 RhoGAP expression vector and the GAP-defective mutant R1255A were obtained from Dr. Ian Macara. The p80 wild type vector and HAT-defective mutant p80mut2 were from Dr. Hendrik Stunnenberg (Nijmegen, Netherlands). The p300CRD1-(14004–1045) mutant DNA was a generous gift from Dr. Neil Perkins (Dundee, UK). The HDAC6 expression vector with a Myc tag was the kind gift from Dr. Tony Kouzarides (Cambridge, UK). The Spi-GLE1-Luc plasmid was from Dr. Peter Haldosen (Karolinska, Sweden). The plasmid pF2A-CREB consisting of the DNA binding domain of Gal4 (residue 1–147) fused with the transcription activation domain of CREB (residue 1–280) and the pFR-Luc plasmid containing luciferase reporter gene were purchased from Stratagene (La Jolla, CA). All plasmids were prepared with the plasmid maxi prep kit from Qiagen (Hilden, Germany).

Cell Culture and Treatment—NIH-3T3 cells were grown at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mm l-glutamine. Unless otherwise indicated, the concentration of IGH was 50 ng/ml. This concentration of GH was within the physiological range for circulating rodent GH (33).

RhoA Activation Assay—Serum-starved cells were stimulated with hGH as indicated and then lysed on ice for 15 min in 1× MLB buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM MgCl2, 1 mM EDTA, 2% glycerol, 500 mM NaF, 1 mM Na3VO4, and 1 tablet of Complete™ protease inhibitor mini mixture per 10 ml). After that the samples were centrifuged at 14,000 × g at 4 °C for 10 min, and the protein concentrations of the supernatants were measured. 900 μg of each supernatant were immediately affinity-purified with 30 μg of GST-rhotekin-RBD fusion proteins freshly precoated to glutathione-agarose beads. The precipitates were washed three times with MLB buffer, and the bound RhoA-GTP was eluted in 20 μl of Laemmli sample buffer. Samples were separated by 12% SDS-PAGE and detected by monoclonal RhoA antibody.

Immunoprecipitation—After treatment as indicated, cells were lysed at 4 °C for 20 min in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na3VO4, and 1 tablet of Complete™ protease inhibitor mini mixture (10 ml)). Cell lysates were centrifuged at 14,000 × g for 15 min, and the supernatants were precleared by protein A/G plus agarose beads on ice for 45 min, and then the protein concentrations of the resulting supernatants were determined. For each immunoprecipitation, 500 or 1000 μg of protein A/G plus agarose for 1 h or overnight. Immunoprecipitates were recovered as three times with P81 buffer (50 mM Tris-HCl, pH 6.7, 2% SDS, and 0.7% β-mercaptoethanol). Blots were then washed for 30 min with three changes of PBST at 22 °C. Efficacy of stripping was determined by re-exposure of the membranes to ECL. Thereafter, membranes were rebloked and immunolabeled as de-
sired. Immunolabeling was detected by the enhanced chemiluminescence kit according to the manufacturer’s instructions.

**Confocal Laser Scanning Microscopy**—Cells were grown on coverslips in complete medium until 40–50% confluence and then transfected with C3 exoenzyme. At 24 h of post-transfection, cells were deprived of serum for 16 h and treated with GH for 30 min. After that the cells were fixed in 4% paraformaldehyde in PBS for 20 min, washed by PBS, and blocked in 2% BBX (0.1% Triton X-100, 0.1% bovine serum albumin, 250 mM NaCl, prepared in PBS). The cells were then incubated with the polyclonal antibody against Stats for 1 h at room temperature. After being washed by BBX, cells were incubated with anti-rabbit IgG conjugated with Cy3. The nonspecifically bound antibody was removed by washing in BBX. Thereafter, DAPI nuclear staining was performed for 5 min. Labeled cells were observed in a Leica DM RXA2 fluorescent microscope. Images were converted to the tagged information file format and processed with the Adobe Photoshop program.

**Nuclear Extraction**—Cells were rinsed once with ice-cold PBS and incubated with 500 µl of buffer A (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 2 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 tablet of Complete™ protease inhibitor mini mixture per 10 ml) at 4 °C for 30 or 60 min. The nuclei were collected by centrifugation at 4,000 rpm at 4 °C for 10 min and then lysed in 40 µl of buffer B (20 mM HEPES, pH 8.0, 400 mM NaCl, 1 mM EGTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 tablet of Complete™ protease inhibitor mini mixture per 10 ml) at 4 °C for 60 min. The nuclei were then centrifuged and the supernatants were collected. The protein concentration were measured and diluted as described below.

**Gel Electrophoretic Mobility Shift Assay**—Nuclear extracts were prepared as described above. The double-stranded Spi-GLE1 probe was labeled by biotin onto the 3’ end according to the manufacturer’s instructions. Briefly, 5 pmol of each complementary oligonucleotide of the probe DNA was labeled by biotin-N4-CTP in the presence of terminal deoxynucleotidyltransferase at 37 °C for 30 min. The reactions were stopped by EDTA. Terminal deoxynucleotidyltransferase was excluded by chloroform/isooamylic alcohol. Then the two completed end-labeling reactions were annealed at room temperature for 1 h. The binding reactions for gel electrophoretic mobility shift assay were performed by prechilled 10% of nuclear extract of each sample with 1 µg of poly(dIdC) in binding buffer (10 mM Tris, 50 mM KCl, 1 mM dithiothreitol, pH 7.5, 0.5 mM EDTA) for 15 min on ice. For supershift analysis, the extracts were incubated with the antibodies against Stat5, RhoA, or p38 for another 10 min on ice. 20 fmol of Spi-GLE1-LUC probe was then added. The binding mixture was incubated at room temperature for 20 min. The samples were separated by electrophoresis on 6% polyacrylamide gel in 0.5× TBE buffer at 120 V at 4 °C, followed by transfer to Hybond-N+ membrane at 380 mA for 30 min. After that, the membrane was blocked for 15 min and incubated with streptavidin-horseradish peroxidase conjugate for another 15 min. After washing, the biotin-labeled DNA was detected by the enhanced chemiluminescence method in accordance with the manufacturer’s instructions. The double-stranded Spi-GLE1 probe was labeled as described above and then subjected to immunoprecipitation by 10 µg of p300 monoclonal antibody or control monoclonal antibody. The immunoprecipitates were washed by lysis buffer and re- suspended in 50 µl of Tris/ATP buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 10 mM magnesium acetate, 100 µM ATP). The kinase reactions were performed in the presence of 1 µg of Elk-1 fusion protein and 200 µM ATP at 30 °C for 30 min. Elk-1 phosphorylation was detected by use of a specific phospho-Elk1 (Ser-383) antibody.

**ROCK Activity Assay**—500 µg of cell lysates per sample were prepared and immunoprecipitated as described previously by ROCK polyclonal antibody. The immunoprecipitates were washed by lysis buffer and re-suspended in 50 µl of Tris/ATP buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 10 mM magnesium acetate, 100 µM ATP). The kinase reactions were performed in the presence of 1 µg of recombinant RhoA and 200 µM ATP at 30 °C for 30 min. ROCK activity was detected by use of a specific phospho-MYPT1 (Thr-696) antibody.

**p300 HAT Activity Assay**—500 µg of nuclear extracts were prepared as described above and then subjected to immunoprecipitation by 10 µg of p300 monoclonal antibody or control monoclonal antibody. The immunoprecipitates were washed three times by PBS and once by assay buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 1 mM dithiothreitol). The enzymatic reactions were performed at 30 °C for 30 min in the presence of 10 µM of core histones and 200 µM acetyl-CoA containing 0.5 µCi of [³H]Acetyl-CoA. 5 µl of each sample was blotted on the P81 paper in triplicate. The paper squares were washed three times in trichloroacetic acid and once in acetone. The radioactivity was measured by scintillation counter. The counts/min of the enzyme sample was subtracted by that of the negative control sample.

**Site-directed Mutagenesis**—RhoA mutation (R188A) was generated by PCR mutagenesis using Quikchange® site-directed mutagenesis kit (Stratagene). The primer pairs are: 5'-CGTTGGAGAAGAAAAGCTGGTGCTGGTTGTGC3' and 5'-AGCAAGGCAACCGCTTCTCCTC3' labeled with 5'-biotin (Roche). The luciferase reporter assay—Either 0.2 µg of the reporter plasmid pFR-Luc and 4 ng of the fusion trans-activator plasmid pFA2-CREB or 0.5 µg of Spi-GLE1-Luc reporter plasmid were transfected with 0.8 µg of the DNA of interest into cells grown in 2% serum containing Dulbecco’s modified Eagle’s medium with 60–80% cell confluence. 0.4 µg of β-galac-tose plasmid were transfected as the transfection efficiency. After 36 h, cells were stimulated with 50 nM GH for 6 h immediately or after chemical pretreatment for 30–60 min. Cells were washed by PBS twice and lysed by RLB buffer (25 mM glycglycine, pH 7.8, 1% Triton X-100, 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol) for 20 min. The luciferase activity was measured in the presence of RAB buffer (25 mM glycglycine, pH 7.8, 15 mM potassium phosphate, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol, and 1 mM ATP) and 200 mM n-luciferin. β-Galactosidase activity was measured in the assay buffer (100 mM sodium phosphate, pH 7.3, 1 mM MgCl₂, 50 mM β-mercaptoethanol, 0.665 mg/ml α-nitrophenyl β-galactopyranoside). The luciferase activities were calculated as the fold of stimulation after normalized by protein concentration and the β-galactosidase activity.

**Statistical Analysis and Presentation of Data**—All experiments were performed at least three times. In the case of Western blot analysis, representative data from one experiment are presented. Numerical data were expressed as mean ± S.D. Data were analyzed using the two-tailed t test or analysis of variance. Results were considered significant at the 5% level.

**RESULTS**

**GH Stimulation of NIH-3T3 Cells Increases the Activity of RhoA**—We utilized the GST-fused rhotekin-RBD as a specific probe to determine the level of RhoA activation in lysates of NIH-3T3 cells stimulated by GH. The rhotekin-RBD probe recognizes only the active GTP-bound form but not the inactive GDP-bound form of RhoA (34). We observed a marked increase in the level of GTP-bound RhoA upon cellular stimulation with GH, persisting from 2 to 30 min after initial GH stimulation, followed by a decline to the basal level 60 min after GH stimulation (Fig. 1A). The GH-stimulated formation of RhoA-GTP was also dose-dependent with an increase in GTP-bound RhoA observed at 5–50 nM GH but not at GH concentrations lower than 0.5 nM (Fig. 1C). GH stimulation of NIH-3T3 cells did not alter the total level of RhoA protein over the examined time periods nor under differential dose conditions (Fig. 1, B and D). Thus, RhoA is a small Ras-like GTPase utilized by GH to exert its effect on cellular function.

**GH-stimulated Activation of RhoA Requires the Kinase Activity of JAK2**—GH activates both JAK2 and c-Src kinases independently of the other (25). We have demonstrated previously that two other small Ras-like GTPases, Rap1 and Rap, require the activity of both c-Src and JAK2 to be fully activated by GH (25). We therefore examined the requirement of JAK2 and c-Src for GH-stimulated activation of RhoA. As observed in Fig. 1E, upon forced expression of the JAK2 kinase-deficient mutant (R882E) (35), the basal level of RhoA-GTP was diminished, and GH-stimulated formation of RhoA-GTP was completely prevented. In contrast, forced expression of a c-Src kinase-inactive mutant (K295R/Y527F) (36), under conditions that prevent GH-stimulated activation of c-Src (25), did not alter the ability of GH to stimulate formation of GTP-bound RhoA. Co-transfection of both JAK2-R882E mutant and c-Src-R295R/Y527F mutant prevented the ability of GH to stimulate the activation of RhoA identical to that observed with forced expression of JAK2-R882E mutant alone. Forced expression of the kinase-deficient mutants of JAK2 and c-Src was verified by Western blot analysis (Fig. 1, G and H), and their expression
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Fig. 1. GH stimulation of NIH-3T3 cells increases the formation of GTP-bound RhoA in a JAK2-dependent manner. A–D, GH stimulates the formation of GTP-bound RhoA in both a time- and dose-dependent manner. NIH-3T3 cells were stimulated with the indicated doses of GH for the indicated times, and the GST-linked probe rhohtekin-RBD, which recognizes only the active GTP-bound form of RhoA, was used to separate RhoA-GTP from the inactive RhoA-GDP. GTP-bound RhoA (A) was visualized by Western blot analysis. Total cellular RhoA (B and D) was also determined in total cell lysates by Western blot analysis. The results presented are representative of a minimum of three independent experiments. E–H, GH-stimulated activation of RhoA requires the kinase activity of JAK2. NIH-3T3 cells were transiently transfected with empty vector or the expression construct containing either kinase-deficient JAK2 (K882E) or kinase-deficient c-Src (K295R/Y527F) or both and stimulated with 50 nM GH for 2 min. The GST-linked probe rhohtekin-RBD, which recognizes only the active GTP-bound form of RhoA, was used to separate RhoA-GTP from the inactive RhoA-GDP. GTP-bound RhoA (E) was visualized by Western blot analysis. Total cellular RhoA (F and H) was also determined in total cell lysates by Western blot analysis. The forced expression of JAK2-K882E (G) and c-Src-K295R/Y527F (H) is indicated (G and H).

did not alter the total protein level of RhoA (Fig. 1F). Therefore, we conclude that the kinase activity of JAK2, but not c-Src, is required for GH-stimulated formation of GTP-bound RhoA.

p190 RhoGAP Inhibits GH-stimulated RhoA Activity—p190 RhoGAP, the most extensively studied GAP for Rho GTPases, regulates the activation state of RhoA through acceleration of GTP hydrolysis (37). We therefore examined the effect of p190 RhoGAP on GH-stimulated RhoA activity. As observed in Fig. 2A, GH-stimulated RhoA activity was dramatically repressed by forced expression of p190 RhoGAP. Forced expression of p190 RhoGAP did not alter the total protein level of RhoA (Fig. 2B). Forced expression of p190 RhoGAP was verified by Western blot analysis (Fig. 2C). p190 RhoGAP is therefore a potent inhibitor for GH-stimulated formation of GTP-bound RhoA.

JAK2-induced Dissociation of RhoA from p190 RhoGAP Is Required for GH-stimulated RhoA Activation—Because both JAK2 and p190 RhoGAP regulated GH-stimulated formation of GTP-bound RhoA, we examined the relationship between these molecules on the ability of GH to stimulate the activation of RhoA. As observed in Fig. 3A, forced expression of JAK2 increased both basal and GH-stimulated RhoA activity as would be expected from the JAK2-dependent activation of RhoA by GH described above. Forced expression of p190 RhoGAP prevented GH-stimulated formation of RhoA-GTP, and the forced expression of p190 RhoGAP concomitant with the forced expression of JAK2 completely prevented the JAK2-enhanced activation of RhoA by GH (Fig. 3A). The replacement of Arg-1283 of p190 RhoGAP by Ala has been reported previously to disrupt GAP activity (6). We therefore utilized this p190R1283A mutant to competitively antagonize the activity of endogenous p190 RhoGAP. In Fig. 3E, forced expression of the p190R1283A mutant robustly enhanced both basal and GH-stimulated formation of GTP-bound RhoA. Forced expression of the kinase-deficient mutant JAK2-K882E prevented GH-stimulated formation of RhoA-GTP, and the forced expression of p190 RhoGAP competed with the forced expression of JAK2 to disrupt GAP activity. The lack of GH-stimulated formation of GTP-bound RhoA as a result of forced expression of JAK2-K882E was reversed by concomitant transfection with p190R1283A. The effect of p190 RhoGAP on GH-stimulated formation of GTP-bound RhoA is therefore exerted downstream of JAK2. Forced expression of p190 RhoGAP, p190R1283A, JAK2, and JAK2-K882E was verified by Western blot analysis (Fig. 3, C, D, G, and H), and the forced expression of these molecules did not alter the total cellular level of RhoA (Fig. 3, B and F). Thus, GH-stimulated formation of RhoA-GTP requires JAK2 kinase activity and subsequent inactivation of p190 RhoGAP.

One mechanism for the regulation of p190 RhoGAP activity is tyrosine phosphorylation (6, 7). We therefore first examined whether GH could affect the level of tyrosine phosphorylation of p190 RhoGAP. p190 RhoGAP was immunoprecipitated from extracts derived from cells stimulated with GH and the immu-
noprecipitates subject to Western blot analysis for phosphotyrosine. As shown in Fig. 3J, GH had no effect on the tyrosine phosphorylation status of p190 RhoGAP despite equivalent amounts of p190 RhoGAP being precipitated (Fig. 3K). In the basal unstimulated cellular state, RhoA and p190 RhoGAP are associated with each other (Fig. 3L). Despite the inability of GH to alter the tyrosine phosphorylation status of p190 RhoGAP, GH stimulation of cells resulted in a dissociation between p190 RhoGAP and RhoA as observed in Fig. 3L. The GH-stimulated dissociation of RhoA and p190 RhoGAP was prevented by
forced expression of the kinase-inactive mutant JAK2-K882E (Fig. 3L). Equivalent loading of p190 RhoGAP is shown in Fig. 3M. Forced expression of JAK2-K882E was verified by Western blot analysis as observed in Fig. 3M. Thus, JAK2 kinase activity is required for GH-stimulated dissociation of p190 RhoGAP and RhoA with the resultant release of RhoA from inhibition by p190 RhoGAP and the formation of GTP-bound RhoA.

RhoA Does Not Affect GH-stimulated Activation of JAK2-p44/42 MAP Kinase Pathway—RhoA has been reported to activate the JAK2/Stat3 pathway through the regulation of JAK2 tyrosine phosphorylation status and activity (14). *Clostridium botulinum* C3 exoenzyme selectively inhibits RhoA activity by the ADP-ribosylation of residue Asn-41 in the RhoA effector domain and has been utilized widely to investigate RhoA function (38, 39). As observed in Fig. 4A, the GH-stimulated activation of RhoA was dramatically repressed by forced expression of C3 exoenzyme. Forced expression of C3 exoenzyme did not alter the total protein level of RhoA (Fig. 4B). Fig. 4B also showed that ADP-ribosylated RhoA migrated slower in comparison to the unmodified species in SDS-PAGE, and this phenomenon has been reported previously (39). Forced expression of C3 exoenzyme was verified by Western blot analysis (Fig. 4C). Thus C3 exoenzyme is a potent inhibitor of GH-stimulated RhoA activity.

We therefore utilized C3 exoenzyme to examine the effect of RhoA on the ability of GH to stimulate JAK2 and p44/42 MAP kinase activities. As observed in Fig. 4D, forced expression of C3 exoenzyme did not alter the level of tyrosine phosphorylation of JAK2 stimulated by GH. The equivalent loading of JAK2 and the forced expression of C3 exoenzyme were demonstrated by Western blot analysis (Fig. 4, E and F). Therefore, RhoA does not participate in GH stimulation of JAK2.
been studied extensively (3, 26, 29). RhoA has also been demonstrated previously (40) to be involved in the stimulation of p44/42 MAP kinase activity in stretch-induced signaling. We therefore examined the effect of forced expression of C3 exoenzyme on GH-stimulated p44/42 MAP kinase activity. GH stimulation of vector-transfected NIH-3T3 cells resulted in a rapid and prolonged activation of p44/42 MAP kinase activity such that 60 min after GH stimulation, p44/42 MAP kinase activity was still higher than in the basal state (Fig. 4G). Forced expression of C3 exoenzyme did not affect the ability of GH to activate p44/42 MAP kinase nor alter the duration of the GH-stimulated increase in p44/42 MAP kinase activity (Fig. 4G). Thus, RhoA activity does not participate in GH stimulation of p44/42 MAP kinase activity.

**GH Stimulates ROCK Activity in a RhoA-dependent Manner**—ROCK is a major RhoA effector molecule mediating the majority of the reported cellular functions of RhoA (11, 12). We therefore examined whether the GH-stimulated formation of GTP-bound RhoA resulted in the activation of ROCK. We observed a time-dependent activation of ROCK kinase activity upon GH stimulation, first observed at 5 min, sustained until 30 min, and then returned to the basal level after 60 min (Fig. 5A). Forced expression of C3 exoenzyme completely prevented the GH stimulation of ROCK activity (Fig. 5A). Equivalent loading of ROCK protein and the forced expression of C3 exoenzyme were demonstrated by Western blot analysis (Fig. 5, B and C). Thus, GH stimulates ROCK activity in a RhoA-dependent manner. As we have demonstrated that GH-stimulated formation of RhoA-GTP requires JAK2 kinase activity and subsequent inactivation of p190 RhoGAP, we further examined the dependence of GH-stimulated ROCK activity on JAK2 and p190 RhoGAP. As observed in Fig. 5D, forced expression of JAK2 robustly increased both basal and GH-stimulated ROCK activity, whereas forced expression of p190 RhoGAP prevented GH-stimulated ROCK activity. When p190 RhoGAP was forcibly expressed concomitantly with JAK2, the JAK2-enhanced activation of ROCK by GH was dramatically inhibited (Fig. 5D). Concordantly, forced expression of the p190R1283A mutant enhanced both basal and GH-stimulated ROCK activity. Forced expression of the kinase-deficient mutant JAK2-K882E prevented GH-stimulated activation of ROCK, and the inhibitory effect of JAK2-K882E on GH-stimulated ROCK activity was reversed by concomitant transfection with p190R1283A (Fig. 5D). Equivalent loading of ROCK protein and forced expression of p190 RhoGAP, p190R1283A, JAK2, and JAK2-K882E were verified by Western blot analysis (Fig. 5, E–G). Therefore, GH-stimulated ROCK activation is elicited by formation of GTP-bound RhoA that requires JAK2 kinase activity and subsequent inactivation of p190 RhoGAP.

**RhoA-ROCK Is Required for GH-stimulated, Stat5-mediated Transcription**—The JAK-Stat pathway is one of the predominant pathways utilized by GH to mediate transcriptional activation (19). GH-stimulated JAK2 activation has been demonstrated to result in the tyrosine phosphorylation and transactivation of both Stat5 isoforms (20). We therefore examined whether RhoA and/or ROCK are required for GH-stimulated, Stat5-mediated transcription by use of a reporter assay specific for both isoforms of Stat5 (41). GH stimulation of NIH-3T3 cells resulted in an approximate 5-fold increase in Stat5-mediated transcription in comparison to the basal state (Fig. 6A). Concommitantly, forced expression of p190 RhoGAP, p190R1283A, JAK2, and JAK2-K882E were verified by Western blot analysis (Fig. 6, B–D). Therefore, GH-stimulated Stat5-mediated transcription is elicited by formation of GTP-bound RhoA that requires JAK2 kinase activity and subsequent inactivation of p190 RhoGAP.
Forced expression of the C3 exoenzyme resulted in a decrease in the basal level of Stat5-mediated transcription and abrogated the ability of GH to stimulate Stat5-mediated transcription. NIH-3T3 cells were transiently transfected with the expression vectors for C3 exoenzyme or kinase-defective mutant ROCK-KD together with Spi2.1-GLE1-Luc and β-galactosidase vector. Cells were treated with 50 nM GH, and GH-stimulated, Stat5-mediated transcription was determined by measuring luciferase activity as described under “Experimental Procedures.” The results were normalized by protein contents and β-galactosidase activity. Data presented are mean ± S.E. of triplicate determinations. Experiments were repeated three times. B–D, RhoA is not required for GH-stimulated tyrosine phosphorylation of Stat5. NIH-3T3 cells were transiently transfected with the expression vectors for C3 exoenzyme and then stimulated with 50 nM GH for the indicated times. Western blot analysis of 50 μg of cell lysates per sample using the monoclonal antibody recognizing tyrosine-phosphorylated Stat5A/B was performed (B). The membrane was stripped and reprobed with anti-Stat5 to demonstrate equal loading of Stat5 proteins (C). The level of forced expression of C3 exoenzyme was shown (D). E, RhoA is not required for GH-stimulated nuclear translocation of Stat5. NIH-3T3 cells were transiently transfected with the expression vectors for C3 exoenzyme and stimulated with 50 nM GH. The samples for confocal laser scanning microscopy were prepared and visualized as described under “Experimental Procedures.” The effect of C3 exoenzyme on GH-stimulated nuclear translocation of Stat5 was shown. Stat5 molecules were labeled with Cy3 indicated as red. C3 exoenzyme was fused with a GFP tag that is indicated as green. Nuclei were stained by DAPI indicated as blue. F and G, RhoA is not required for GH-stimulated DNA binding of Stat5. NIH-3T3 cells were transiently transfected with the expression vectors for C3 exoenzyme and stimulated with 50 nM GH. The nuclear extracts for gel electrophoretic mobility shift assay were prepared and were subject for reactions as described under “Experimental Procedures.” The effect of C3 exoenzyme on GH-stimulated DNA binding of Stat5 is shown (F). The level of forcefully expressed C3 exoenzyme is shown in G, H–J, RhoA is not required for GH-stimulated degradation of Stat5. NIH-3T3 cells were transiently transfected with the expression vector for C3 exoenzyme and then stimulated with 50 nM GH. Samples were collected 2 or 6 h after GH treatment, and Western blot analysis of 50 μg of cell lysates per sample was performed by using the monoclonal antibody recognizing tyrosine-phosphorylated Stat5A/B (I). The membrane was stripped and reprobed with anti-Stat5 to demonstrate equal loading of Stat5 proteins (I). The forced expression of C3 exoenzyme is shown in J. The results presented are representative of a minimum of three independent experiments.
acutely stimulate tyrosine phosphorylation of Stat5 (Fig. 6B) over 5–60 min. Forced expression of C3 exoenzyme was demonstrated by Western blot analysis and did not alter the total cellular level of Stat5 (Fig. 6, C and D). Similarly, forced expression of C3 exoenzyme did not alter the nuclear translocation of Stat5 in response to GH as determined by confocal laser scanning microscopic analysis (Fig. 6E). Furthermore, as observed in Fig. 6F, forced expression of C3 exoenzyme did not affect GH-stimulated binding of Stat5 to its DNA-response element (SPI-GLE1). Binding of Stat5 to SPI-GLE1 was verified by supershift analysis with a specific Stat5 antibody (Fig. 6F). The expression of C3 exoenzyme was demonstrated by Western blot analysis (Fig. 6G). Neither RhoA nor p300, a reported transcriptional regulator of Stat5, was contained in the GH-stimulated complex binding to SPI-GLE1 (Fig. 6F). It has been demonstrated previously that c-Cbl-mediated degradation of Stat5 abrogates GH-stimulated tyrosine phosphorylation, nuclear translocation, or DNA binding of Stat5 (42). Such degradation resulted in reduced levels of tyrosine-phosphorylated Stat5 within 2–6 h after GH stimulation (42). Forced expression of C3 exoenzyme did not alter the amount of tyrosine-phosphorylated nor total Stat5 within 6 h of GH stimulation (Fig. 6H). Thus, RhoA regulates GH-stimulated, Stat5-mediated transcription through a mechanism other than initial activation and DNA binding of Stat5 or degradation of activated Stat5.

**PKA Inhibits GH-stimulated, Stat5-mediated Transcription through Inactivation of RhoA**—GH has been reported to stimulate CREB phosphorylation and CREB-mediated transcription during adipocytic differentiation (43, 44). In addition, PKA/CREB has been observed previously (45) to modulate Stat5-mediated transcription in erythroid cells. Furthermore, PKA has been demonstrated to reduce the activity of RhoA by phosphorylation of RhoA on serine residue 188 (46). We therefore examined whether RhoA would be the pivot for potential cross-talk between the PKA and JAK2-Stat5 pathways. We observed that GH stimulation of NIH-3T3 cells did not affect PKA activity, phosphorylation of CREB, nor CREB-mediated transcription, however, forskolin was able to stimulate all three above-mentioned events (data not shown), suggesting that NIH-3T3 cells utilized in this study possess a functional PKA/CREB pathway that is not responsive to GH stimulation. However, as shown in Fig. 7A, 8-Br-cAMP or forskolin abrogated the formation of GTP-bound RhoA stimulated by GH. Conversely, a myristoylated PKA-specific inhibitor enhanced the GH-stimulated formation of GTP-bound RhoA (Fig. 7A). We proceeded to examine the effect of modulation of PKA activity on the ability of RhoA to enhance GH-stimulated, Stat5-mediated transcription. As observed in Fig. 7C, forskolin abrogated, and PKA-specific inhibitor markedly enhanced, the magnitude of GH-stimulated, Stat5-mediated transcription. Concordantly, forced expression of the mutant RhoA S188A, not subject to inhibition by PKA, dramatically increased GH-stimulated, Stat5-mediated transcription (Fig. 7C). Forskolin failed to prevent the enhancement of GH-stimulated Stat5 transcriptional activity observed with forced expression of RhoA S188A, indicating that PKA required serine residue 188 of RhoA to inhibit GH-stimulated Stat5 activity (Fig. 7C). Consistently, the enhancement of GH-stimulated, Stat5-mediated transcription observed in the presence of the PKA inhibitor was completely prevented by forced expression of the C3 exoenzyme inhibiting RhoA (Fig. 7C). We therefore conclude that RhoA is the pivot...
for cross-talk between PKA and GH-stimulated, Stat5-mediated transcription.

**p300 Inhibits GH-stimulated RhoA-mediated Stat5 Transcriptional Activity by Recruiting HDAC6—**p300 is a transcriptional cofactor with histone acetylase activity that may either activate or repress transcription (31, 32, 47). Indeed, p300 has been demonstrated previously to enhance prolactin-stimulated, Stat5-mediated transcription (49). Previous studies (17, 18) have suggested that RhoA regulates transcription via repression of p300/CBP. We therefore examined the potential role of p300 in the effect of RhoA on GH-dependent Stat5-mediated transcription. Forced expression of p300 reduced the basal level of Stat5-mediated transcription and completely abrogated GH-stimulated, Stat5-mediated transcription (Fig. 8A). We next examined whether the histone acetylase activity of p300 was required for repression of GH-stimulated, Stat5-mediated transcription. HAT activity was not affected by cellular stimulation with GH nor was it altered by forced expression of the C3 exoenzyme (Fig. 8B). We also utilized the p300mutAT2 with 6 amino acids mutated in the HAT domain with resultant defective HAT activity (50). Concordantly, the inhibitory effect of p300 was independent of HAT activity, as forced expression of p300mutAT2 also dramatically abrogated GH-stimulated, Stat5-mediated transcription (Fig. 8A). We next examined whether the CRD1 domain of p300 was required for repression of GH-stimulated, Stat5-mediated transcription. The CRD1 domain is responsible for the repressor activity of p300 through SUMO modification-dependent recruitment of HDAC6 (32). Forced expression of the p300CRD1 domain mutant (p300ΔCRD1) did not significantly repress GH-stimulated, Stat5-mediated transcription (Fig. 8A). Further evidence that the repressor ability of p300 on GH-stimulated, Stat5-mediated transcription was due to its association with histone deacetylase activity was provided by the observation that the deacetylase inhibitor, trichostatin A (TSA), reversed the abrogation of GH-stimulated, Stat5-mediated transcription as a consequence of forced expression of p300 (Fig. 8A). Forced expression of HDAC6, the histone deacetylase associating with the CRD1 domain of p300, also dramatically abrogated GH-stimulated, Stat5-mediated transcription (Fig. 8C). The inhibitory effect of the forced expression of HDAC6 on GH-stimulated, Stat5-mediated transcription was also reversed in the presence of TSA (Fig. 8C). Forced expression of the p300ΔCRD1 mutant prevented inhibition of GH-stimulated, Stat5-mediated transcription as a consequence of forced expression of HDAC6 (Fig. 8C), indicating that HDAC6 association with p300 is required for repression of GH-stimulated, Stat5-mediated transcription.

We next examined the involvement of p300 in the RhoA-dependent enhancement of GH-stimulated, Stat5-mediated transcription. As shown in Fig. 8D, forced expression of p300 eliminated the enhancement of GH-stimulated, Stat5-mediated transcription as a consequence of forced expression of RhoAS188A. Conversely and concordant with the above observation, forced expression of p300ΔCRD1 relieved GH-stimulated, Stat5-mediated transcription from the suppression consequent to forced expression of the C3 exoenzyme (Fig. 8D). p300 is therefore downstream of the effect of RhoA on GH-stimulated, Stat5-mediated transcription.

**DISCUSSION**

Multiple members of the family of small Ras-like GTPases have been demonstrated to participate in the cellular effects of GH (24, 25, 26, 28). Ras itself and RaIA are required for GH-stimulated activation of p44/42 MAP kinase and subsequent Elk-1 mediated transcription (25, 26). Rap1 constrains the activity of GH-stimulated p44/42 MAP kinase through inactivation of RaIA (24). GH also stimulates Rac activation to regulate actin cytoskeleton rearrangement and cell motility (28). As shown in Fig. 8, the forced expression of RhoA enhanced GH-stimulated, Stat5-mediated transcription. As shown in Fig. 8, the forced expression of p300A188A also enhanced GH-stimulated, Stat5-mediated transcription (Fig. 8A). Forced expression of p300A188A also enhanced GH-stimulated, Stat5-mediated transcription as a consequence of forced expression of HDAC6 (Fig. 8A), indicating that HDAC6 association with p300 is required for repression of GH-stimulated, Stat5-mediated transcription (Fig. 8). For further evidence that the repressor ability of p300 on GH-stimulated, Stat5-mediated transcription was due to its association with histone deacetylase activity was provided by the observation that the deacetylase inhibitor, trichostatin A (TSA), reversed the abrogation of GH-stimulated, Stat5-mediated transcription as a consequence of forced expression of p300 (Fig. 8A). Forced expression of HDAC6, the histone deacetylase associating with the CRD1 domain of p300, also dramatically abrogated GH-stimulated, Stat5-mediated transcription (Fig. 8C). The inhibitory effect of the forced expression of HDAC6 on GH-stimulated, Stat5-mediated transcription was also reversed in the presence of TSA (Fig. 8C). Forced expression of the p300ΔCRD1 mutant prevented inhibition of GH-stimulated, Stat5-mediated transcription as a consequence of forced expression of HDAC6 (Fig. 8C), indicating that HDAC6 association with p300 is required for repression of GH-stimulated, Stat5-mediated transcription.

We next examined the involvement of p300 in the RhoA-dependent enhancement of GH-stimulated, Stat5-mediated transcription. As shown in Fig. 8D, forced expression of p300 eliminated the enhancement of GH-stimulated, Stat5-mediated transcription as a consequence of forced expression of RhoAS188A. Conversely and concordant with the above observation, forced expression of p300A188A relieved GH-stimulated, Stat5-mediated transcription from the suppression consequent to forced expression of the C3 exoenzyme (Fig. 8D). p300 is therefore downstream of the effect of RhoA on GH-stimulated, Stat5-mediated transcription.

**FIG. 7.** PKA inhibits GH-stimulated, Stat5-mediated transcription through inactivation of RhoA. A and B, PKA inhibits GH-stimulated RhoA activity. NIH-3T3 cells were pretreated with 500 μM 8-Br-cAMP, 20 μM forskolin, or 10 μM PKA inhibitor for 30 min before stimulating with 50 nM hGH for 2 min. The GST-linked probe rhotekin-RBD, which recognizes only the active GTP-bound form of RhoA, was used to separate RhoA-GTP from the inactive RhoA-GDP. GTP-bound RhoA (A) was visualized by Western blot analysis. Total cellular RhoA (B) was also determined in total cell lysates by Western blot analysis. C, PKA inhibits GH-stimulated, Stat5-mediated transcription via phosphorylation of serine residue 188 of RhoA. NIH-3T3 cells were transiently transfected with the expression vectors for either RhoAS188A mutant or C3 exoenzyme together with Spi2.1-GLE1-Luc and β-galactosidase vector. Cells were pretreated with either 20 μM forskolin or 10 μM myristoylated PKA inhibitor as indicated for 30 min prior to stimulation with 50 nM GH for 6 h. GH-stimulated, Stat5-mediated transcription was determined by measuring luciferase activity as described under “Experimental Procedures.” The results were normalized by protein contents and β-galactosidase activity. Data presented are mean ± S.E. of triplicate determinations. Experiments were repeated three times.

Here we have identified another small GTPase, RhoA, as a regulator of GH-stimulated, Stat5-mediated transcription in NIH-3T3 cells.

The activity of RhoA is tightly controlled by both positive regulators GEFs and negative regulators GAPs and GDIs (2). We have demonstrated here that GH stimulation results in disassociation of p190 RhoGAP from RhoA in a JAK2-dependent manner with subsequent activation of RhoA. Redistribution of p190 from the cytosol to a detergent-insoluble fraction has been demonstrated previously (8) to be involved in the activation of RhoA by epidermal growth factor and oncogenic H-RasV12. Phosphorylation of p190 RhoGAP is not required for redistribution of p190 RhoGAP nor activation of RhoA (8). We also observe that although GH stimulation results in a disassociation of p190 RhoGAP and RhoA, GH does not stimulate the
tyrosine phosphorylation of p190 RhoGAP. However, other studies have reported that the GAP activity of p190 can be regulated by Src-dependent tyrosine phosphorylation (6, 7). In this regard it is interesting to note that GH stimulation of the formation of GTP-bound RhoA is entirely JAK2-dependent. This is despite the observation that GH also stimulates Src activity in this cell line (25) and predominantly utilizes Src for the activation of RalA and Rap1 (24, 25). GH-dependent JAK2 activity necessary for the RhoA-RhoGAP dissociation may be required for the phosphorylation of other molecules potentially involved in the activation of RhoA. For example, cytokine-stimulated tyrosine phosphorylation of SOCS-3 results in prolonged activation of Ras due to binding of p120RasGAP with tyrosine phosphorylated SOCS-3 (53). Whether an analogous mechanism exists for GH-stimulated JAK2-dependent activation of RhoA remains to be determined.

We have demonstrated here that activation of RhoA is required for GH-stimulated, Stat5-mediated transcription. We observed that the effect of RhoA was not due to altered tyrosine phosphorylation, nuclear translocation, DNA binding, nor degradation of Stat5. However, it has been reported recently (16) that an oncogenic mutant of RhoA promoted tyrosine phosphorylation and DNA binding activity of Stat5A by a JAK2-dependent mechanism. Concordant with the lack of effect of RhoA on GH-stimulated tyrosine phosphorylation of Stat5, we also observed no effect of GH-activated RhoA on JAK2 tyrosine phosphorylation required for its activity and tyrosine phosphorylation of Stat5. The oncogenic form of RhoA is therefore
exhibiting a different spectrum of activity compared with the wild type molecule under physiological stimulation. Differential functioning of oncogenic mutants of other signaling molecules compared with the wild type form has been reported previously (54–56). For example, oncogenic Ras constitutively activates phospholipase D by a protein kinase C-independent mechanism that is different from the protein kinase C-dependent mechanism used by wild type Ras (56). Instead, we have observed that GH-activated RhoA increases GH-stimulated, Stat5-mediated transcription by abrogation of p300/HDAC6 repression of Stat5-mediated transcription.

In principle, transcription requires the DNA be accessible to transcription factors and RNA polymerase. However, chromatin structure impedes such access (29). Therefore, although ligand-stimulated phosphorylation and subsequent DNA binding of transcription factors is required for transcriptional activation, it is not sufficient to initiate transcription. Chromatin remodeling and function of transcriptional cofactors and components of the basal transcription machinery are all required to ensure proper transcription initiation (29). p300 and CBP, a family of transcription cofactors with intrinsic histone acetyltransferase (HAT) activity, play a key role in the regulation of promoter activity by many transcription factors including p53, NF-κB, AP1, and Stats (14, 57). Here we demonstrate that p300 inhibits GH-stimulated Stat5 transcription. Although p300 has been regarded as transcription co-activator, it has also been demonstrated to possess repressor ability (30–32). It has been reported that p300 inhibits p53-mediated transcription of the Bax promoter (32). p300 also mediates the down-regulation of c-Myc, and this effect does not require the HAT activity of p300 (31). Most interesting, we also observed that the inhibitory effect of p300 on GH-stimulated, Stat5-mediated transcription is independent of HAT activity as the HAT activity-deficient p300mutAT2 mutant also inhibited GH-stimulated, Stat5-mediated transcription. Furthermore, GH did not stimulate HAT activity. p300 also acts as a scaffold for the assembly of multiple cofactor complexes. This function can either activate or inactivate transcription, as determined by the property of the protein(s) recruited by p300 (30). It is noteworthy that an HDAC activity is required for p300 to diminish Stat5 transcriptional activity, suggesting that p300 may assemble with an HDAC. Indeed, a CRD1 (cell cycle regulatory domain 1) region on p300 has been demonstrated recently to be crucial for recruitment of HDAC6, and this event is the mechanism of the transcription-repressive effect of p300 on p53 (32). Concordantly, we have demonstrated here that the inhibition of GH-stimulated, Stat5-mediated transcription by p300 is attributed to CRD1-mediated recruitment of HDAC6. HDAC6 is one member of the HDAC family that includes 11 members (58). HDACs convert chromatin to a condensed state by catalyzing deacetylation of histones and thus serve as a key mechanism for transcriptional repression (58). HDAC6 has been demonstrated to inhibit the transcription of NF-κB and Runx (59). It has been suggested recently that the recruitment of HDAC6 by p300 is achieved by sumoylation of the CRD1 domain on p300 (32). Both HDAC6 and p300 are substrates for the ubiquitin-related SUMO modifier (32, 60). SUMO attaches to the lysine residues of the target protein in a way analogous to that of ubiquitination; however, unlike ubiquitination, sumoylation does not accelerate protein degradation but mediates protein-protein interaction, subcellular compartmentation, and protein stability (61). Thus SUMO serves as the bridge between HDAC6 and CRD1 of p300. HDAC1 and HDAC4 can also be modified by SUMO (62); however, only HDAC6 can interact with sumoylated CRD1 of p300 (32), indicating that HDAC6 functions exclusively in p300-mediated transcriptional repression.

We further demonstrate that p300 mediates the effect of RhoA on GH-stimulated, Stat5-mediated transcription. The antagonistic relationship between RhoA and p300 has been...
established recently by accumulating evidence. It has been reported that RhoA inhibited whereas its negative regulator, RhoGDI, stimulated CBP/p300-mediated estrogen receptor-dependent transactivation (18). Concordantly, RhoA down-regulates inducible nitric-oxide synthase expression via inhibition of CBP/p300 (17). The mechanism of RhoA inhibition of p300 is still unclear. However, as a serine/threonine kinase, the RhoA effector ROCK may affect the activity of p300 via phosphorylation. p300 is phosphorylated in both quiescent and proliferating cells presumably through cyclin-dependent kinases (64), whereas CBP can be phosphorylated by PKA, CaM-dependent kinase N, and p44/42 MAP kinase (30). Little is known of how phosphorylation affects p300/CBP functions, although phosphorylation of p300 appears to increase its HAT activity (65). It is also possible that an intermediate molecule is the substrate of phosphorylation which in turn affects p300 activity. Indeed, it has recently been demonstrated that phosphorylation of Elk-1 enhances its interaction with p300 with resultant activation of p300 (66). It is possible that GH-stimulated activation of ROCK results in phosphorylation of p300 or a p300-interacting protein so as to disrupt the recruitment of HDAC6 by CRD1, and thus release Stat5-mediated transcription from p300/HDAC6-mediated repression. The precise mechanism by which RhoA/ROCK regulates p300, and whether phosphorylation participates in this event, requires further elucidation.

Here we have observed that cAMP/PKA inhibits GH-stimulated, Stat5-mediated transcription, but GH itself does not affect the PKA-CREB pathway. Interaction between PKA and the cellular effects of GH has been reported previously. For example, it has been demonstrated that the cAMP/PKA pathway mediates the effects of GH in ovarian granulosa cells by up-regulation of the protein level of PKA itself by GH (67). cAMP also potentiates the ability of GH to prime preadipocytes for differentiation and simultaneously stimulate the phosphorylation and activation of CREB (43). However, the GH-stimulated activation of CREB was reported to be independent of PKA (43), and it has not been addressed whether the effect of cAMP on GH-primed differentiation is mediated through activation of CREB. Thus, cAMP/PKA modulation of GH signaling does not necessarily require GH-dependent regulation of the PKA-CREB pathway. Instead, we have identified that the effect of cAMP/PKA on GH-stimulated, Stat5-mediated transcription requires serine residue 188 in RhoA, thus mediating repression of RhoA activity. It has been demonstrated previously (46, 48, 63) that cAMP/PKA antagonizes RhoA/ROCK activity via phosphorylation of serine residue 188, which possibly increases the squelching of RhoA by its negative regulator RhoGDI. One recent study (27) has further demonstrated an antagonistic effect of PKA on RhoA/ROCK-mediated gene expression. It has been reported that prostaglandin E2 and stem cell factor enhance erythropoietin-mediated Stat5 transactivation by the PKA-CREB-CBP/p300 pathway (45, 51). This apparent discordance between the above stimulating effect of PKA on Stat5 transcriptional activity and our findings that PKA inhibits Stat5 may be due to the different signaling pathways utilized by PKA upon different ligand stimulation in a different cellular context. In our system, PKA does not utilize CREB (data not shown) to regulate GH-stimulated, Stat5-mediated transcription. Thus, PKA may divergently regulate Stat5 transactivation by different mechanistic pathways, and the selection is determined by the specific cellular conditions.

In summary, we demonstrate here that small GTPase RhoA and its effector serine/threonine kinase ROCK are activated by growth hormone through JAK2-dependent dissociation of RhoA from its negative regulator p190 RhoGAP. GH utilizes RhoA and ROCK to abrogate the repression of Stat5-mediated transcripion by HDAC6 recruited by p300, thereby dramatically enhancing GH-stimulated, Stat5-mediated transcriptional activity. We also demonstrate that PKA inactivates RhoA through serine residue 188 that consequently suppresses GH-stimulated, Stat5-mediated transcription. A diagram summarizing this RhoA-dependent pathway to regulate Stat5-mediated transcription is provided in Fig. 9. We have therefore provided a novel mechanism by which GH-stimulated activation of Ras-like small GTPases regulates Stat5-mediated transcription stimulated by GH. The involvement of HDAC activity in GH-stimulated gene transcription also raises the possibility of direct GH participation in epigenetic modification of gene expression (52).

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